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THE BRITISH JOURNAL
OF
EXPERIMENTAL PATHOLOGY

THE ÆTIOLOGY OF EPIDEMIC INFLUENZA: EXPERIMENTS IN SEARCH OF A FILTER-PASSING VIRUS.

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THE severity and wide distribution of epidemic influenza during the past two years has resulted in much discussion as to the ætiology of the disease, and has directed a large amount of effort towards the elucidation of that problem. From some areas the reported bacteriological findings* [1, 2] in clinical and post-mortem material have been interpreted as supporting Pfeiffer's belief that *B. influenzæ* is the causal agent. In other places this organism has been found in a much smaller proportion of cases [3, 4], and the discrepancy has been enough to cast some doubt upon the theory that it is the primary factor. That the differences in findings were not altogether due to differences in *personnel* and technique is indicated by comparing results, obtained during the epidemic in Toronto in the spring of this year, with those of the same men using the same technique working in England during the previous year. *B. influenzæ* was almost constantly present in the cases in England, but was found in only 24 per cent. of similar cases during the 1920 outbreak in Toronto. A glance over the

* A complete critical review of the literature upon the ætiology of influenza by Fildes and McIntosh appears in this Journal, vol. i, pp. 119 and 159.

recent literature will show that the pneumoccocus, the various streptococci and other bacteria varied in a similar way, and it therefore seems reasonable to regard these organisms, including *B. influenzæ*, as secondary invaders which vary in predominance in different localities, and in the same locality at different times.

Rosenow ([5, 6]) claims to have isolated from cases of influenza a green-producing streptococcus which has pandemic characteristics, and as a result of animal experiments he draws certain analogies between the lesions produced by this organism and the pathological findings in human influenza. His work has not yet been confirmed by other observers.

It is perhaps fair to state that the trend of opinion has gradually been in favour of the theory that the primary infecting agent is a filter-passer.

Keegan ([7]), however, reports a complete absence of symptoms in nine volunteers who were inoculated with filtered sputum and nasal washings from influenza patients. Two patients were used, one in the second and one in the fourth day of the disease. The nasal washings (75 c.c. from each) were mixed with the sputum, shaken and then filtered. Half a cubic centimetre of the filtrate was sprayed into the nose of each volunteer, four being used for one patient and five for the other. They were isolated for ten days and had no symptoms.

Rosenau ([8]), in extensive experiments, carried out on a large number of volunteers, found that no symptoms occurred in the subjects after they had been inoculated with large amounts of nasopharyngeal and bronchial secretions, or with whole blood, from cases of typical influenza. Similar inoculations with suspensions of Pfeiffer's bacillus also failed to produce any symptoms.

Nicolle and Lebailly ([9]) report symptoms of influenza in two volunteers who had been inoculated subcutaneously with filtered sputum from influenza patients; also in two monkeys inoculated under the conjunctiva and by nasal instillation with the same sputum unfiltered.

Dugarric de la Rivière ([10]) caused himself to be inoculated subcutaneously with filtered blood from influenza patients, and as a result became ill with the typical symptoms of influenza. He considered that this afforded him protection, as no symptoms resulted from spraying his throat and nasopharynx with filtered influenzal sputum on the tenth day after the first inoculation.

Yamanouchi, Sakakami and Iwashima ([11]), by nasopharyngeal inoculation of volunteers with influenzal sputum, both filtered and unfiltered, produced typical symptoms of the disease in eighteen out of twenty-four subjects. The six failures were the only ones in the series who had previously had influenza, and it was therefore presumed that they had immunity. Filtered blood from influenza patients, administered in the same way, and subcutaneous inoculation of filtrates from blood or sputum, produced similar results. A pure culture of Pfeiffer's bacillus and mixed cultures of pneumococci

streptococci, staphylococci and the cocci common in sputum sprayed into the throat and nose of fourteen healthy people did not cause any illness.

Olitsky and Gates [12], by intratracheal injection of filtered bronchial and nasopharyngeal secretions from influenza cases during the first thirty-six hours of the disease, produced in rabbits a condition resembling the pathological change of influenza in man.

Further evidence in support of this view has been furnished by Bradford, Bashford and Wilson [13] and Gibson, Bowman and Connor [14]. The former describe a minute, filter-passing organism which they found constantly in cases of influenza, and which they were able to grow by special cultural methods. After inoculation with subcultures they record symptoms and lesions in monkeys, and especially in guinea-pigs, which are noted as typical of the disease in man. The organisms were recovered in pure culture from the animals.

Gibson, Bowman and Connor independently isolated an organism which both groups of workers considered identical with that described by Wilson. They found "typical experimental influenzal lesions" in monkeys, rabbits, guinea-pigs and mice after inoculation with cultures, and recovered the organism from the tissues of the animals. Earlier in their work, before they had obtained cultures, the same typical lesions were seen in animals after the introduction of blood and sputum from influenza patients, and, by reinoculation of filtered extracts from abnormal experimental lungs, the same pathological change was repeated in rabbits and guinea-pigs up to the second reinoculation.

It seemed to us desirable that further evidence should be obtained as to the ætiological relationship of a filter-passing organism to influenza, and this investigation was commenced with that object in view.

OBSERVATIONS ON THE PATHOGENICITY OF INFLUENZAL FILTRATES.

The patients from whom we obtained our material were all typical cases of influenza from the epidemic of 1919–1920. They were in the wards of the service of Dr. Duncan Graham, Professor of Medicine, and for the most part were in the earlier stages of the disease.

The various materials obtained from these cases during life or after death were filtered through thoroughly tested Berkefeld or Mandler filters. Such filtrates were always bacteriologically tested.

Guinea-pigs were largely used for the inoculations by the intra-peritoneal route. Rabbits and mice were used less often. All animals were kept under the best hygienic conditions and were killed for autopsy by a sharp blow on the back of the neck.

In general it was found that all animals examined a few days after inoculation with these products showed marked hæmorrhagic lesions in the lungs but no gross changes in other organs. A filtrate prepared from such lungs or from the blood of the same animal reproduced the same lesions in a second animal, and, indeed, these could be carried through eight passages.

Findings in Inoculated Guinea-pigs.

Since most of the work was done with guinea-pigs, it will be necessary to discuss in some detail the findings after inoculating these animals. We have already mentioned the fact that when they were killed a few days after injection, hæmorrhagic lesions in the lungs were found in a considerable number.

The first striking feature noted about the animals was that they did not become ill. The temperature of all our inoculated animals was carefully taken for a time and no abnormalities found; and it soon became apparent that we could not judge the probability of pulmonary lesions from watching the behaviour of the pig.

The essential feature of the typical lesion was the peculiar type of hæmorrhagic change. This was patchy in distribution, it extended into the lung substance and could be seen on section. On the surface it appeared in varying forms. Sometimes the whole lung was almost covered with small red areas 2 to 3 mm. in diameter. Sometimes these were more sparsely scattered, or they might be confined to one or two lobes. The larger lesions appeared to result from coalescence of the smaller ones, and were often 10 to 15 mm. in diameter. The centre of such an area was usually a dark, deep red colour and the periphery, diffuse and irregular in outline, was a brighter red. Sometimes a large part of one lobe was solidly involved in this change, and when this was true it was frequently the part nearest the root of the lung. There was never any fluid exudate or fibrin in the pleural cavity.

A few flecks of red on the surface was not considered abnormal. Neither was a greyish, glistening, slightly raised appearance that had a small and irregular distribution, nor small, pale, slightly raised areas that occurred irregularly. These were found frequently in pigs killed for other purposes.

Two other more extensive atypical lesions were also seen, sometimes in pigs with no previous inoculation, as well as in inoculated animals with or without hæmorrhagic change. They were considered to have no connection with the typical change. In one sort the lung was hard and the surface uneven. It was a yellowish grey colour, and hard yellowish nodules 2 to 3 mm. in diameter showed through the surface. Usually a whole lobe was involved, frequently an upper one, and sometimes two or three lobes were affected. Microscopically it was seen to be a chronic inflammatory change. The air-space was completely obliterated, and the picture was that of the organisation of a previous pneumonic condition.

The lung of the other type was smaller in size than normal. It appeared solid and homogeneous and had a dull salmon-pink colour. Frequently the small posterior lobe was alone affected, sometimes small parts of two or three lobes near the hilum, or perhaps only an irregularly shaped part of one lobe. The gross appearance gave one the impression of a congenital atelectasis.

The cultures of the organs made at the autopsy were in most cases sterile. However, a small proportion of the hæmorrhagic lungs gave positive cultures, the predominating organisms being small Gram-negative bacilli that did not produce gas from glucose and streptococci. *B. coli* was found in a few cultures from abdominal organs.

The filtrates, with very few exceptions, were sterile. The failures were not due to faulty filters, but probably to contamination from the air in handling the filtrate. A large Gram-positive bacillus and a large Gram-negative bacillus were the only organisms found. These were evidently not responsible for the lesions in the inoculated animals, for such contaminations were very rare (as may be seen from the protocols cited), and the same lesions were present when the inoculant was sterile.

Nor were the hæmorrhages due to the protein injected. The animals had never been treated before; they were not sensitised; they did not exhibit any anaphylactic symptoms, and the appearance of the lung was not that of a protein lesion ([15]). Gross changes in the other organs were very uncommon and are described later. The main interest centred in the typical pulmonary lesions, and the following table shows the incidence of animals in which they were found and the inoculant associated with them.

TABLE I.—*Showing the Numbers of Guinea-pigs Inoculated and the Number that had Pulmonary Lesions.*

Inoculant used.	Number of animals inoculated.	Number that showed gross pulmonary lesions.	Number with no gross lesion in the lungs.
Patient's whole blood	31	10	21
Patient's filtered red blood-corpuscles	27	17	10
Patient's filtered plasma	18	8	10
Filtered empyema fluid	4	4	0
Patient's filtered nasal washings	2	0	2
Filtered human tracheal mucous membrane	2	1	1
Filtered positive guinea-pig lung	66	35	31
Filtered blood from positive guinea-pig	11	6	5
Whole blood of positive guinea-pig	43	25	18
Laked blood of positive guinea-pig	6	2	4
Unfiltered lung of positive guinea-pig	15	8	7
Unfiltered lung of negative guinea-pig	3	1	2
Filtered lung of negative guinea-pig	12	3	9
Whole blood of negative guinea-pig	11	8	3
Filtered influenzal sputum	14	9	5
Unfiltered influenzal sputum	2	2	0
Totals	267	139	128

After one type of inoculant only did we fail to find pulmonary hæmorrhages, viz. filtered nasal washings. But the number of animals used in this instance was so small that no conclusion can be drawn. If more had been inoculated we probably would have found hæmorrhages in some. Typical hæmorrhages were present after patients' whole blood, sputum and filtrates from blood-corpuscles, plasma, empyema fluid and tracheal mucous membrane. Considered altogether, approximately half of the guinea-pigs had typical lesions.

Reviewing the results in animals inoculated directly from each of the seventeen cases, it is seen that some guinea-pig lesions were associated with every patient except one, and that approximately half of the total number of pigs inoculated were affected. The following table shows this in detail:

TABLE II.—*Showing the Numbers of Guinea-pigs Inoculated from the various Influenza Cases and the Number that had Pulmonary Lesions.*

Name of patient.	No. of animals inoculated.	No. positive.	No. negative.
Ba'd.	14	5	9
Ba't.	4	2	2
Be'l.	4	1	3
Di'n.	13	9	4
Gr'n.	2	2	0
Gr'g.	3	3	0
Ki'e.	6	2	4
Le'l.	4	2	2
Ma's.	4	2	2
Ra'n.	17	11	6
Ro'y.	4	0	4
Sh'i.	4	2	2
Sh'n.	2	2	0
Autopsy.	1	0	1
Is'd.	7	2	5
Ro'n.	4	2	2
Co's.	5	4	1
Ma'n.	2	2	0
Totals	100	53	47

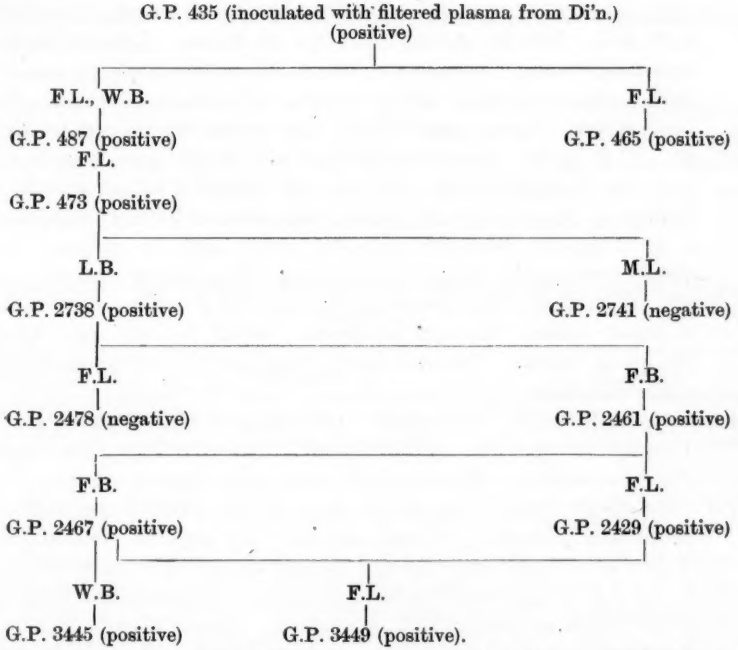
After reinoculation the same pathological picture appeared for several repetitions. In one instance it was found after seven reinoculations, once after six, once after five, and twice after three. From our subsequent experience, it seems certain that by using large series of animals this repetition of lesions could have been carried on indefinitely. In only two series did reinoculation fail completely. Frequently both the blood and lung of one animal were used to inoculate two, and we therefore sometimes had four or five animals an

equidistant number of reinoculations removed from the one patient. As a rule, the more animals used each time the more chance there was of continuing, for if one failed to show hæmorrhages another might do so. There was never an element of surety that reinjection of a pathological lung would reproduce the same picture in the next animal. Sometimes blood was successful, sometimes lung, and sometimes both failed.

The length of time the animal was allowed to live did not seem to be a factor, for typical lesions were found as early as two and as late as nineteen days.

The following chart illustrates a typical series of reinoculations in guinea-pigs :

CHART 1.



W.B. = whole blood. L.B. = laked blood. F.B. = filtered blood. F.L. = filtered lung. M.L. = mashed lung.

The following are the protocols relating to Chart I.

PROTOCOL I.

19 : 2 : 20. G.P. 435. Inoculated with 3 c.c. filtered plasma from Di'n. Filtrate sterile. No sign of illness. Killed 4 days after injection. Lung : many bright red hæmorrhagic areas. Heart's blood, lung, liver, spleen : sterile.

23 : 2 : 20. G.P. 487. Inoculated with 3 c.c. whole blood of G.P. 435. No sign of illness. Killed 3 days after injection. Lungs: marked and very numerous hæmorrhagic areas throughout. Lungs "stand up" and are speckled with these small hæmorrhages, from 1 to 3 mm. in diameter, and a few larger. Heart's blood, lung, liver, spleen: sterile.

23 : 2 : 20. G.P. 465. Inoculated with filtered lung from G.P. 435. Filtrate sterile. No sign of illness. Killed 4 days after injection. Lungs mottled with brownish dull red areas and pale raised patches. These areas vary much in size. Spleen slightly enlarged. Heart's blood, spleen, liver: sterile. Lung: large Gram-positive bacillus.

26 : 2 : 20. G.P. 473. Inoculated with one-half of filtrate from lung of G.P. 487. Filtrate sterile. No sign of illness. Killed 5 days after injection. Lung: numerous reddish-brown hæmorrhagic areas with dark centre and diffuse red periphery. Heart's blood sterile. Lung, liver, spleen: Gram-positive cocci in pairs and chains, non-hæmolytic.

2 : 3 : 20. G.P. 2738. Inoculated with 3 c.c. whole blood from G.P. 473 + 7 c.c. distilled water. No sign of illness. Killed 5 days after injection. Lung: thickly spotted with areas of hæmorrhagic change, 3 to 6 mm. in diameter, seen on surface and on section. Liver: deeply congested. Heart's blood, lung, liver, spleen: sterile.

2 : 3 : 20. G.P. 2741. Inoculated with lung of G.P. 473 mashed in sterile distilled water. No sign of illness. Killed 5 days after injection. No gross lesions. Heart's blood, lung, spleen, liver: small Gram-positive bacillus.

2 : 3 : 20. G.P. 2478. Inoculated with filtered lung from G.P. 2738. Filtrate sterile. No sign of illness. Killed 6 days after injection. No gross lesions. Heart's blood, lung, liver, spleen: sterile.

7 : 3 : 20. G.P. 2461. Inoculated with 3 c.c. blood from G.P. 2738 laked and filtered. Filtrate sterile. No sign of illness. Killed 7 days after injection. Lung: thickly spotted with areas of hæmorrhage 3 to 4 mm. in diameter. Some are confluent and form larger areas; seen on section and on surface. Spleen slightly large. One hæmorrhagic area, 4 × 6 mm., on large bowel. Heart's blood, lung, liver, spleen: sterile.

14 : 3 : 20. G.P. 2467. Inoculated with 4 c.c. blood from G.P. 2461 laked and filtered. Filtrate sterile. No sign of illness. Killed 7 days after injection. Lungs: on two lobes there are hæmorrhagic areas, about 1 cm. in diameter, with dark red centre and diffuse brighter red periphery. Heart's blood, lung, liver, spleen: sterile.

14 : 3 : 20. G.P. 2429. Inoculated with filtered lung from G.P. 2461. Filtrate sterile. No sign of illness. Killed 7 days after injection. Two or three small red flecks on surface; not considered patho-

logical. No other gross change. Heart's blood, lung, liver, spleen: sterile.

21 : 3 : 20. G.P. 3445. Inoculated with 5 c.c. whole blood from G.P. 2467. No sign of illness. Killed 8 days after injection. Lungs: few small hæmorrhagic areas scattered throughout. No other gross lesions. Heart's blood, lung, liver, spleen: sterile.

21 : 3 : 20. G.P. 3449. Inoculated with filtered lungs of G.P. 2467 and G.P. 2429. Filtrate sterile. No sign of illness. Killed 8 days after injection. Lungs: three hæmorrhagic areas extending into the lung substance. Heart's blood, lung, liver, spleen: sterile.

Charts 2 and 3 with the accompanying protocols show similar results and have been omitted.

Looking for corroborative evidence of the infective nature of the inoculant, on two occasions we heated the filtered plasma and filtered corpuscles at 56° C. for one hour before injecting them, and at the same time inoculated an equal number of animals with unheated filtrates from the same patient. The following table shows the comparison of the results from each:

TABLE III.—*Showing the Results from Injecting Heated and Unheated Filtrates.*

Name.	Inoculant.	Unheated.			Heated.		
		Number of animals.	Number positive.	Number negative.	Number of animals.	Number positive.	Number negative.
Be'l.	Filtered plasma	2	1 (10 days)	1 (7 days)	2	1 (8 days) 1 (9 days)	0
	Filtered corpuscles	2	0	1 (7 days) 1 (10 days)	2	0	1 (8 days) 1 (9 days)
Sh'i.	Filtered plasma	2	0	1 (8 days) 1 (8 days)	2	1 (8 days)	1 (10 days)
	Filtered corpuscles	2	1 (19 days) 1 (19 days)	0	2	1 (8 days) 1 (19 days)	0
Totals		8	3	5	8	5	3

It is certain that heating the inoculant to the extent noted did not diminish the lesions. In fact more hæmorrhages occurred after heating, which indicates that some pigs with heated filtrate had lesions when those with corresponding unheated filtrate had none.

Plasma from three convalescent patients was tested for any power it might possess to inhibit the lesions found after inoculation of filtrates.

The following is a brief note of the patients from whom they were obtained:

A. (Ra'n.). Patient became ill February 12th, 1920; had high fever and consolidation of lung. Temperature continuously normal from February 20th, 1920. Blood removed on March 5th after patient had left hospital.

B. (He'y.). Patient became ill March 8th, 1920; had fever, but no pulmonary consolidation; was markedly ill. Temperature continuously normal March 15th. Blood taken March 18th, 1920.

C. (M. Sm'h.) First ill March 1st, 1920; had pulmonary consolidation and severe illness. Temperature continuously normal March 16th. Blood taken March 18th.

The plasma from these three samples of blood was filtered and mixed with filtrates from pathological animals in amounts varying from 3 to 5 c.c. These mixtures were incubated at 37° C. for one hour (air) before inoculation.

Control injections of the same filtrates, without plasma, and the convalescent plasma alone were always made at the same time.

Altogether eleven guinea-pigs were inoculated, and the results failed to show that the convalescent plasma had any effect in preventing the pulmonary lesions. In one instance hæmorrhages were seen after inoculation with a filtrate from a hæmorrhagic lung incubated with plasma, while the animal injected with the same amount of filtrate alone had none.

Furthermore, convalescent serum was incubated with an influenzal patient's whole blood for one hour and injected subcutaneously into two guinea-pigs (in amounts of 5 c.c.), one of which was killed in four days and showed a few scattered hæmorrhages throughout both lungs. The other was killed in thirty-one days, and in its lungs were found numerous fairly large consolidated areas, which proved on microscopic examination to be of the proliferative type.

The gross lesions in other organs were very infrequent, being noted only six times in a series of 159 animals. They were in detail as follows: hæmorrhage into wall of bowel, twice; slight hæmorrhage on surface of liver, twice; hæmorrhage 8 × 10 mm. into rectus abdominis muscle, once; extensive retroperitoneal hæmorrhage in lower abdomen, once. The spleen was sometimes slightly enlarged. These occurred so seldom that their significance was not regarded as important when compared with the frequency of pulmonary hæmorrhage, which was undoubtedly the typical lesion of the condition with which we were dealing.

Both the organs with gross lesions and those apparently normal were studied microscopically.

Lung.—The microscopic changes throughout the series were of two types —hæmorrhagic and proliferative. The former corresponded to the gross and so-called "typical" lesion; the latter was not always apparent to the naked eye. These may for convenience be described separately, although they sometimes occurred together.

The proliferative lesion will be considered first. In very many sections the alveolar walls were thickened, due to proliferation of endothelial cells. Where the change was least the outline of the alveoli could easily be traced, but the air-space of the lung was considerably lessened. In places of greater change single alveoli were completely obliterated by the proliferation; others near by were only a small fraction of their original size, and the outline of the alveolar walls was lost in the diffuse mass of cells. Where this was most marked the tissue was solid and the air-space entirely obliterated. The cells were rather large and irregular in outline; the nuclei were large, central, oval or round, and stained with moderate intensity. The cell masses included capillaries. Giant-cells were occasionally seen. A recent thrombosis of a small branch of a pulmonary artery was associated with this type of lesion.

Although some lungs showed only proliferation, it was common to find hæmorrhage as well. Its situation was variable. It was sometimes small in amount and irregular in distribution, situated interstitially in the thickened alveolar wall or in a mass of cells; but it was also extensive in some areas, filling the partially obliterated alveoli and infiltrating the interstitial tissue between them.

The picture presented by this combination did not suggest that the two processes had a common cause, or that one resulted from the other. Both the hæmorrhage and the proliferation occurred alone so definitely and so frequently that their association appeared to be accidental, and could not be construed as signifying a more fundamental relationship. Further, where they did occur together, in no instance was hæmosiderin present in the endothelial cells. This fact strongly confirms the view that the lesions were independent of each other.

The hæmorrhage was undoubtedly the most singular and striking lesion. It was this that in the gross attracted one's attention, and was described in the articles ([13, 14]) already mentioned as typical of "experimental influenza" in animals, and closely comparable to the early lesions of influenza in the human lung. After reading these accounts we were satisfied that the hæmorrhages we had seen were the same lesions that they reported, and in fact one of the workers already quoted ([14]), having examined our specimens in our laboratory, agreed in this opinion.

The amount of lung involved in the hæmorrhage varied considerably, as may be judged from the description of the gross appearance. The minutest lesion was a capillary hæmorrhage into the alveolar wall, but it was always just an incident in a more extensive change. In most sections the hæmorrhage appeared in small patches. In these the alveoli were completely filled with

blood and stood out sharply defined from air-holding tissue. Where they coalesced to form a massive hæmorrhage large sections of the lung were airless, except for a few scattered empty alveoli that were prominent because of contrast. In many sections blood was seen in the bronchi, and in a very few places slight œdema was observed. A peculiar feature was the lack of leucocytic reaction. Several times there was a recent thrombosis of a branch of the pulmonary artery. The endothelial proliferation, as we have pointed out, could not be regarded as a response to the extravasated blood. Neither in the hæmorrhage itself, nor in the alveolar walls that trabeculated it nor around the margin of the area was any excess of leucocytes seen. The thrombi, however, did contain a moderate number of polymorphonuclear leucocytes.

The thick and irregular muscular coat of the arteries, the folded epithelium of the bronchioles and the masses of lymphocytic cells in the peribronchial tissue are normal structures in the guinea-pig's lungs.

The microscopic lesions in other organs were not frequent, but the following were noted:

Myocardium.—Congestion, without any degeneration or œdema. Slight round-cell infiltration about some of the smaller arteries. Several small diffuse hæmorrhages. The muscle-fibres were broken in these areas, but there was no leucocyte reaction.

Kidney.—Varying degrees of congestion, sometimes interstitial, sometimes affecting the glomeruli only. There was no degeneration nor exudate in the tubules. In one case small areas of interstitial œdema were present alongside the focal interstitial congestion.

Liver.—Marked congestion was occasionally seen. Associated with this small focal hæmorrhages were present in one instance, with diffuse œdema in the portal fibrous tissue, but complete absence of leucocyte reaction.

Spleen.—A few sections show congestion.

Findings in Inoculated Mice.

Fifty-six white mice were inoculated intraperitoneally—with the exception of a few subcutaneously—with material from six cases of influenza. The plan of procedure and technique outlined above for guinea-pigs was followed for these animals. Although some mice had hæmorrhagic lungs we considered it impossible to deduce any experimental evidence from the findings, because a very large percentage of animals yielded cultures of ordinary bacteria. It was common to find organisms from the heart's blood, and the other organs were very frequently infected. The flora of these cultures was not studied fully.

Findings in Inoculated Rabbits.

Six rabbits were inoculated intravenously and one intraperitoneally with filtrates of plasma or corpuscles from four influenza patients. Of these, four

had no gross lesions, one had a few small hæmorrhages in the lungs, one had a small hæmorrhage into the tracheal mucous membrane, and one hæmorrhage into the trachea and œdematous and congested lungs. Fourteen cubic centimetres of the whole blood of this rabbit was injected intravenously into another. It showed one small hæmorrhage in the lung. There were no other gross lesions. None of the animals were ill.

The microscopic study of the lungs showed lesions strictly similar to those described for guinea-pigs. There was both endothelial proliferation and hæmorrhage. The latter was not so massive as in guinea-pigs and more œdema was present, although this was not a prominent feature.

OBSERVATIONS ON THE CULTURE OF A FILTER-PASSING ORGANISM.

An attempt to cultivate a "virus" from filtrates was made by employing a special technique.

A small piece of fresh, sterile rabbit's kidney was put in the bottom of a test-tube (1·5 × 15 cm.) and sterile ascitic fluid added to the depth of about 3 cm. This was incubated for twenty-four hours to ensure sterility, and was stored in the ice-chest until used. Tubes a week old were discarded. After inoculation more ascitic fluid was added until the column was 8 to 9 cm. high. On top of this was poured in about 1 cm. of melted vaseline, which had been previously sterilised. Incubation was carried on at 37·5° C.

In this manner we cultured filtered plasma and filtered corpuscles from three cases (Ba't., Ro'y. and Ki'e.), filtered hæmorrhagic lungs from four guinea-pigs, and filtered nasal washings from one patient (Ki'e.). Each was inoculated into three different samples of ascitic fluid and thorough controls were made.

The cultural appearances of filtered corpuscles were constant and differed from the others. In twenty-four to forty-eight hours a faint diffuse cloudiness appeared in the lower 2 cm. of the tube. This gradually spread throughout the fluid, and was followed by a general increase in opacity. In two to four days a very finely granular greyish deposit appeared in a streak along the side of the tube and to a slight extent in the bottom. This was very noticeable because the quantity was so large.

The other cultures became faintly cloudy in the lower third of the tube in three to five days. Sometimes this became diffuse, sometimes a very finely granular deposit appeared in small amounts about the kidney tissue or for a short distance up the sides of the tube.

As far as we could determine, these appearances did not indicate the growth of an organism, for tubes inoculated with filtered corpuscles and filtered plasma from one of us who had never had influenza changed in exactly the same way. Moreover films of the cultures, fixed in methyl alcohol and stained for fourteen to eighteen hours with Giemsa's stain, and also with Gram's stain, did not reveal the presence of any organisms.

Examinations were made up to the end of four weeks.

It was, however, decided to inject some of the cultures into animals, and the following are the results of this procedure:

Two guinea-pigs, 3747 and 3733, were each given intraperitoneally 3 c.c. of a three-day-old culture of filtered corpuscles from Ba't. After six days no typical lesions were found. Pigs 7477 and 7461 were similarly inoculated with 3 c.c. and 2 c.c. respectively of a five-days-old culture of filtered corpuscles from Ki'e. Both were killed on the fifth day and had no gross lesions.

A five-days-old culture of filtered corpuscles from Ro'y. was used on two pigs—No. 7444 was given 2 c.c. and No. 7488 2·5 c.c. The former, killed after five days, had extensive hæmorrhagic change in the lungs. The filtered lung was injected into pig No. 3732, which was killed after five days and had no gross lesions. Pig No. 7488 was normal at the end of forty-eight hours.

One pig was inoculated with a twenty-three-days-old culture of a filtrate from a hæmorrhagic guinea-pig's lung. It was killed nineteen days afterwards and showed marked pulmonary hæmorrhage.

These results are very far from indicating the causal agent in influenza, and in itself this cultural work is far too meagre to exclude a filter-passing organism as the ætiological factor. We wish, however, to record the technique, and to point out that in two animals the typical pulmonary lesion was found at five and nineteen days after inoculation with apparently sterile cultures.

That this lesion was due to some invisible virus in the culture was not an impossible view, but it seemed a very improbable one. This attitude was further strengthened by the results observed after ordinary broth cultures of filtrates were injected into animals.

The following table summarises these results:

TABLE IV.—*Summary of Results from Inoculation of Broth-cultures of Filtrates.*

Inoculant.	Name.	Number of guinea-pigs inoculated.	Number positive.	Number negative.
12-day culture of filtrate from hæmorrhagic guinea-pig lung	Di'n.	1	1 (8 days)	—
23-day ditto	Le'l.	1	—	1 (19 days)
48-day „	Ra'n.	1	—	1 (7 days)
8-day „	Sh'n.	1	1 (6 days)	—
28-day culture of filtered blood from a positive guinea-pig	—	—	—	1 (9 days)

These cultures were those made as a routine test of sterility of all our filtrates. The older ones had been purposely kept in the incubator in order to correspond to the long incubation usually associated with filterable organisms, but we were unable to demonstrate organisms of any sort in them. Inoculation was intra-peritoneal.

Two animals had typical lung lesions. The filtered lungs of each of them were transferred to two other pigs, and these showed more marked lesions than the first pair—in thirteen days and eight days respectively.

The pathological changes in all of these "positive" animals was exactly the same as that already described.

If we regard it as due to some invisible "virus," we must consider this "virus" to have grown in two widely different types of culture, to have been active in cultures as young as five days and as old as twenty-three, and from different types of cultures to have produced the same sort of lesion in animals at five and twenty-nine days after inoculation. In view of these conditions it seems much more reasonable to believe that the lesions did not result from the injection of the cultures.

TABLE V.—*Showing the Results from Inoculating Guinea-pigs with Filtrates of Material from Healthy People.*

Name.	Diagnosis.	Inoculant.	Number of animals Inoculated.	Number positive.	Number negative
Sh'g.	Fractured leg	Plasma	2	—	2
		Corpuscles	2	—	2
Br'n.	Fractured arm	Plasma	2	1	1
		Corpuscles	2	1	1
Mo'n.	Supra-orbital neuralgia	Plasma	1	—	1
		Corpuscles	3	3	—
Ca'e.	Inguinal hernia	Plasma	2	—	2
		Corpuscles	2	—	2
Hi'g.	Tuberculous adenitis of neck	Plasma	3	2	1
		Corpuscles	3	2	1
Ga'h.	Injury to musculo-spiral nerves	Plasma	2	1	1
		Corpuscles	2	1	1
Mu'y.	Injured perinæum	Plasma	3	2	1
		Corpuscles	2	1	1
H.B.M.	Well	Plasma	2	1	1
		Corpuscles	1	—	1
Ra'u.*	Well	Plasma	2	—	2
		Corpuscles	2	—	2
Wi'i.	Asthmatic bronchitis	Laked whole blood	2	2	0
	Totals		40	17	23

* Blood removed just after convalescence from severe influenza, thirteen days after the temperature first became continuously normal.

CONTROL EXAMINATIONS OF HEALTHY PEOPLE.

As a matter of comparison we inoculated a series of guinea-pigs with filtrates from the corpuscles and plasma of healthy people. The technique was exactly the same as that used for influenza patients. The healthy controls were for the most part people in the surgical wards of the hospital, and none of them had ever had influenza, nor did they develop the disease within a reasonable time after our examination. The results are summarised in Table V, p. 277.

The character of the lesions in these animals agreed in every way with those found after injection of influenzal material. The positive animals shown in the table were those which had typical hæmorrhagic lung changes. Their number was almost half of the total inoculations. Comparing this with the similar table from influenza cases, and considering the small series on which this is based, the percentage of positive animals may be regarded as approximately the same, whether the material injected came from healthy people or influenza patients.

After adding this evidence to that obtained from inoculation with cultures, it was quite apparent that none of the animal lesions had any connection with the infective agent of influenza.

OTHER CONTROLS.

This conclusion was fully justified by the subsequent examination of guinea-pigs that had never received any inoculation.

Three lively and apparently healthy pigs, about 200 grm. weight, were chosen at random from the breeding-pens. Two were killed by a blow on the head and one by cutting the throat. All had very extensive and typical hæmorrhagic change.

The lungs of twenty-three pigs, bled for complement, were also examined. They had previously been used for testing diphtheria antitoxin but appeared healthy when killed. Three of them had hæmorrhages.

These findings revealed the fact that we were dealing with some pathological condition present in our stock pigs. Furthermore we found that not only were our animals affected but those from other laboratories also. Part of our supply came from a farm on the outskirts of the city, and in transit they were sometimes kept for a few days in another laboratory in another building. Four pigs, from a batch that came to us in this way, were killed by a blow on the head immediately they arrived. Two had the typical hæmorrhagic lesion.

DISCUSSION.

Neither the proliferative type of pneumonia nor the hæmorrhagic lesions herein described appear to have received notice in the literature upon spontoneous animal disease. The pneumonia in guinea-pigs attributed to infection with *B. bronchisepticus* by Theobald Smith ([16]) seems to have been an acute

process, with necrosis of the parenchymal cells and infiltration of the area with both endothelial and polymorphonuclear cells. It is possible that the proliferative change we have found may be a late stage of the same disease, but this seems very unlikely. Smith described the involved area of lung in the late stage as "firm, greyish and shrunken to quite small dimensions." He also mentions that the earliest stages of the disease are not definitely known, and it may be that this is what we have observed.* Another possibility that suggests itself is that this proliferative change is due to the dust and particles of *débris* which must of necessity be breathed into the respiratory tract by the guinea-pig by reason of its environment in the ordinary methods of rearing these animals under the usual artificial conditions.

The typical hæmorrhagic lesions described also lack an adequate explanation at the present time. Hæmorrhages in various localities are frequently encountered by every observer experimenting upon animals by inoculating with pathogenic organisms. In such cases, however, one is never in doubt as to the meaning of such lesions, since not only is a typical cellular reaction observed about the area, according to the length of time elapsing after the inoculation and the localisation of the infecting agent, but even in the earliest stages microscopic sections through the area of hæmorrhage will reveal the actual presence of the bacteria in or about the capillaries involved. Moreover, appropriate cultural methods will recover the organism from the lesion.

None of these conditions obtained in the type of hæmorrhage under discussion, since the inoculants did not contain any of the known pathogenic bacteria. It would, however, seem reasonable to expect the usual reaction to infection about such areas if an infecting organism were the cause, and if sufficient time had elapsed for such processes to take place. In almost every instance the red blood cells appeared so fresh and took up oxygen so readily that one was led to think that the hæmorrhage was probably very recent. That it might have been due to the method of killing the animals has been considered. About 300 have been killed by a blow on the back of the head and approximately half of them have had the hæmorrhagic lesion. Records of twenty-four guinea-pigs killed by rapid bleeding from the jugular and carotid vessels show that four had hæmorrhages.

Further investigation is needed to settle the exact ætiology of these lesions, and the questions briefly discussed above will form the basis of a further communication in an attempt to throw some light upon them.

SUMMARY AND CONCLUSIONS.

There is considerable evidence in the recent literature for attributing epidemic influenza to a primary infection with a filter-passing organism. The

* *B. bronchisepticus* was recovered from some of our apparently healthy animals killed for controls, and they may, as carriers, have developed some immunity towards this organism which would account for a low grade of tissue response to infection with it.

most extensive experiments by animal inoculations have been carried out by Bradford, Bashford and Wilson, and Gibson, Bowman and Connor, who reported having found such an organism in tissues and exudates, and obtained it in culture. By inoculating animals, principally guinea-pigs, with filtrates of material from patients and with cultures, they produced "typical experimental influenzal lesions," and recovered the organism in culture from the animals. They also succeeded in transferring the typical lesions from one animal to another. Bradford and Wilson, in a note appended to a criticism by Arkwright ([17]), later retracted their claim that a filter-passing organism had been grown in pure culture, and stated that degeneration forms of bacteria and protein particles in their cultures had been mistaken for such an organism. This retraction may also be applied to the work of Gibson, Bowman and Connor, since they state that they were in agreement with Capt. Wilson that their "organism" was the same as he had found.

We have investigated, with a technique similar to theirs, seventeen cases of influenza, and by inoculating animals, principally guinea-pigs, with filtrates of material from these patients have observed the same typical lesions. By reinoculating one guinea-pig from another we have found the lesions repeated through seven reinoculations.

Heating the inoculant to 56° C. for one hour did not diminish the animal lesions, nor did incubation with convalescent plasma.

We were not able to obtain cultures of a filterable organism, although the attempt to do so was not exhaustive.

We did find, however, that the *typical* lesions appeared in guinea-pigs after inoculation with *sterile* cultures of widely different types and ages.

Further, this "typical" lung change was found in guinea-pigs from our stock and breeding-pens that had never been inoculated, and also in animals that came to us from a breeding-farm and another laboratory.

The same percentage of animal lesions occurred after inoculation with material from healthy controls as with influenzal material.

The lesions under discussion are microscopically of two broad types, proliferative and hæmorrhagic, and have been described in the papers previously quoted. We have pointed out, however, that these two types of tissue change apparently bear no fundamental relationship to each other, and were probably only accidentally associated. They were undoubtedly not produced by the infective agent of epidemic influenza.

The proliferative type is to be regarded as a spontaneous animal disease, the bacteriology of which is being investigated. The hæmorrhagic type of pulmonary lesion is peculiar, inasmuch as there is a striking absence of cellular or other reaction about the areas involved. Its ætiology is obscure, and further work is being done in an endeavour to find an adequate explanation of its occurrence.

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THE EFFECT OF SURGICAL ANÆSTHESIA ON THE REACTION OF THE BLOOD.

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It has been shown by Leathes (1), Collip and Backus (2) and others that variations in respiratory activity or in the rate of lung ventilation are reflected at once in the partition of acid and basic phosphates secreted by the kidney, and to a less extent in the rate of elimination of urinary ammonia. Thus Leathes (1) has attributed the morning alkaline tide in the urine to increased respiratory activity on waking, and it would similarly follow that the high degree of acidity of the night urine is due to a lessened degree of lung ventilation during the hours of sleep. Leathes (1) has shown that the carbon dioxide content of the alveolar air is higher during sleep than it is during the waking hours, while Collip (3) has demonstrated that there is no increase, and in some instances that there is an actual decrease, in the bicarbonate content of the blood of the sleeping individual. This points to an actual decrease in the C_H of the blood during sleep. This latter phenomenon must therefore be considered in the explanation of the highly acid urine secreted during sleep.

It has been assumed, since surgical anæsthesia causes a decrease in the alkali reserve of the blood, that the induction of anæsthesia tends toward a condition of acidosis. As has been shown by the theoretical considerations of L. J. Henderson (4) and the experimental work of Hasselbalch (5), Haggard and Henderson (6), Collip and Backus (2), Grant and Goldman (7), and others, a low level of plasma bicarbonate is in itself no indication of an increased C_H of the blood. It is therefore apparent that the C_H of the blood during anæsthesia, or in fact at any time, can only be determined when the ratio of L. J. Henderson (), $\frac{CO_2 \text{ dissolved}}{CO_2 \text{ combined}}$, is known. Owing to the difficulties in the way of obtaining a reliable sample of alveolar air from an anæsthetised person who is undergoing a major surgical operation, it is very difficult to decide whether the C_H of the blood is altered while a patient is under an anæsthetic.

As the observations of Henderson, Haggard and Coburn (6) have demonstrated the beneficial effects of the inhalation of 6 or 8 per cent. of carbon dioxide in the air by patients following major surgical operations, it might be

expected that the effect of anæsthesia is not to raise the C_H of the blood, but rather to lower it.

Baldwin ([9]) has pointed out that the urine becomes more acid during anæsthesia. Reimann and Bloom ([10]) found that the total acetone bodies of the blood increased during anæsthesia, while Reimann and Hartman ([11]) found that the total acidity of the urine was increased following anæsthesia. They concluded that small but very definite changes in metabolism take place during anæsthesia, and that the reduction of blood bicarbonate which occurs as a result of anæsthesia is due to these changes and not to over-ventilation, as suggested by Henderson and Haggard ([12]).

In order to determine if the partition of the acid and basic phosphates of the urine secreted during anæsthesia would throw any light on this question a series of unselected cases were studied.

The operations were all performed late in the morning. The patient was instructed to empty the bladder at eight o'clock on the morning of the operation. The urine passed just previous to anæsthetisation was collected and the time noted. As soon as the patient was returned to the ward after operation the bladder was catheterised, the time noted, and the sample preserved. A third specimen of the urine was collected as soon as the patient expressed the desire to urinate. The specimens were shaken with a few drops of chloroform as soon as collected and then carried to the laboratory for analysis. The total volume of each sample was noted, and the rate of secretion for each period calculated in terms of cubic centimetres secreted per hour. Analyses were made on 10 c.c. of the various specimens. The relative acidity and the concentration of phosphates were determined by the method of double titration, using methyl orange and phenolphthalein as indicators, as outlined by Leathes ([1]), and later used by Collip and Backus ([2]). The acidity of the specimen determined in this manner denotes the ratio of acid to basic phosphate. Thus an acidity of 70 per cent. means that 70 per cent. of the total phosphates are present as acid salt, while 30 per cent. are present as basic salt. Ammonia was estimated by the formol titration method. The results are expressed in tabular form in Table I. The rate of secretion of urine before, during and after anæsthesia and the rate of secretion and the concentration of ammonia and phosphates for like periods are herein expressed.

The average duration of anæsthesia was one hour. From fifteen to thirty minutes must be added to this to give the time of secretion of the second specimen. This was due to the delay necessarily ensuing in moving the patient from the ward to the operating theatre and back again. The anæsthetics used were in all instances but one ethyl chloride and ether. Nitrous oxide and oxygen was used in this one instance.

The results show that there is considerable variation in the various factors determined in the different cases. Out of fifteen cases, nine showed a slight increase in the rate of secretion of urine during anæsthesia over that manifested

immediately prior to the induction of anæsthesia; one showed no change, while in five the rate was decreased. The acidity of urine or the ratio of acid phosphate to total phosphate was increased in fourteen out of fifteen cases, while in one case this factor was decreased very slightly. The rate of secretion of ammonia was increased to a slight amount in nine cases and diminished in six cases. The rate of phosphate elimination was just noticeably decreased in ten cases and increased in five cases. Results such as these would indicate little or no alteration in the reaction of the blood during anæsthesia. They do not in the least suggest a condition of alkalosis.

There is undoubtedly, in most major surgical operations, increased lung ventilation, which would naturally lead to the manifestation of typical symptoms of temporary alkalosis, such as the disappearance of alkali from the blood by way of the tissues and the urine, unless such reactions were counteracted by some other factor. It is possible that two processes are involved which are mutually antagonistic, and that in the majority of cases they more or less counteract one another in such a way that no appreciable change in blood reaction is brought about. These processes, which are mutually antagonistic, might be termed "the acapnial process" and the "acidotic process of Haggard and Henderson."(13) Increased lung ventilation during anæsthesia would tend to lower the bicarbonate content of the blood, while metabolic disturbances due directly to the anæsthetic would, by the production of acid substances, also tend to lower blood bicarbonate. These two processes when met with separately are, as Haggard and Henderson (13) have pointed out, the direct opposite of each other, although their effect on the blood bircarbonate is, from a superficial view-point, the same. When, however, they are met with simultaneously in the same individual, the one process must necessarily combat or neutralise the other.

To put this theory to the test: a dog was etherised and a cannula placed in the left ureter. A tracheal cannula was then inserted and the anæsthetic given by the Woulff bottle method. The animal was undisturbed for two hours. The urine which had been secreted during this time was then titrated as described above, and found to have an acidity of 50 per cent. Violent hyperpnœa was then induced by intermittent faradic stimulation of the sciatic nerve. This was continued for two hours. The urine secreted during the period of hyperpnœa was then titrated and found to have an acidity of 40 per cent.

Attempts were also made to determine the carbon dioxide content of the alveolar air of dogs during anæsthesia at the same time that the concentration of blood bicarbonate was estimated by the method of Van Slyke and Cullen.(14) The alveolar carbon dioxide tension was determined approximately by a modification of the Plesch (15) method. The animal was allowed to breathe for thirty seconds into a rubber football bladder containing 600 c.c. of expired air. The air in the bladder was then passed through the

Table Showing the Effect of Anæsthesia upon the Urine.

Subject.	Rate of secretion of urine per hour before.	Ditto—during.	Ditto—after.	Acidity of urine before.	Ditto—during.	Ditto—after.	Rate of NH_3 secretion (mgrm. per hr.) before.	Ditto—during.	Ditto—after.	Concentration of NH_3 (mgrm. per 100 c.c.) before.	Ditto—during.	Ditto—after.	Rate of phosphate* excretion before.	Ditto—during.	Ditto—after.	Concentration of phosphate* before.	Ditto—during.	Ditto—after.	Remarks.
1	90	35	30	10	67	68	13·5	12	12	15	35	40	27	23	17	30	66	56	Gas-oxygen anæsthesia.
2	60	20	30	70	74	70	30	10	18	50	50	60	21	7·6	12·6	35	38	42	Abdominal scar removed.
3	40	15	25	70	65	67	12	4	8	30	25	34	14	5·7	10·5	35	38	42	Appendectomy.
4	20	20	45	68	70	80	26	1[illegible]	54	130	90	120	25	20	67·5	125	100	150	Nerve-graft.
5	55	15	15	75	80	80	70	28·5	28·5	140	190	190	55	16·6	30	100	111	200	Hernia.
6	55	20	30	30	80	76	16·5	19	25·5	30	95	85	33	24	42	60	120	140	Varicocele.
7	30	15	35	60	84	80	31·5	13·5	40	105	90	115	21	12	42	70	80	120	Arthrodesis.
8	40	60	20	10	15	60	8	10	14	20	17	70	16	24	20	40	40	100	Hæmorrhoids.
9	25	35	30	50	60	70	10	10·5	10·5	40	30	35	12·5	14	24	50	40	80	Appendectomy.
10	20	35	10	55	70	70	24	30	13	120	85	130	26	39	16	130	111	160	Hæmorrhoids and septum.
11	35	85	—	38	48	—	24	72	—	68	85	—	30	80	—	85	94	—	Inguinal hernia.
12	40	45	—	54	74	—	32	33	—	80	94	—	28	22	—	70	50	—	Varicose veins.
13	55	30	—	8	77	—	14	18	—	26	60	—	14·3	15·6	—	26	52	—	—
14	40	35	—	58	74	—	29	21	—	73	60	—	20	13·3	—	50	38	—	—
15	50	25	—	50	67	—	27·5	32	—	55	130	—	25	19	—	50	75	—	Bone graft.
16	—	75	20	—	80	74	—	22·5	12	—	30	60	—	22·5	11·4	—	30	57	Cranial defect.

* Phosphate is expressed in terms of c.cs. of $\frac{N}{10}$ acid required to carry 100 c.c. of urine from the phenolphthalein to the methyl orange point.

apparatus of Fridericia and the percentage of carbon dioxide determined. The method is not entirely satisfactory, but the results are nevertheless suggestive. A protocol of such an experiment follows:

Dog, ♂, 16 kilo.

2.25 p.m.	Under ether.				
2.30 „	Blood-plasma from femoral vein $CO_2 = 42{\cdot}4$.				
2.40 „	Ureters cannularised.				
2.50 „	Tracheal cannula inserted.				
3.00 „	Alveolar air	$CO_2 =$ 5·2 per cent.			
3.00 „	Blood-plasma	$CO_2 = 35$	„	Ratio $\frac{CO_2 \text{ dissolved}}{CO_2 \text{ combined}}$	$= 0{\cdot}0852$.
3·05 „	Alveolar air	$CO_2 =$ 5·2	„		
4.05 „	Left kidney secreting.				
4.30 „	Alveolar air	$CO_2 =$ 4·0	„		
4.30 „	Blood-plasma	$CO_2 = 28$	„	Ditto.	$= 0{\cdot}0819$.
5.30 „	Alveolar air	$CO_2 =$ 4·85	„		
5.30 „	Blood-plasma	$CO_2 = 28$	„	„	$= 0{\cdot}0841$.
6.00 „	Alveolar air	$CO_2 =$ 4·25	„		
6.00 „	Blood-plasma	$CO_2 = 28$	„	„	$= 0{\cdot}0834$.
8.30 „	Alveolar air	$CO_2 =$ 4·25	„		
8.30 „	Blood-plasma	$CO_2 = 28$	„	„	$= 0{\cdot}0834$.

Urine secreted 70 per cent. acid. 130 mgrm. NH_3 per 100 c.c.

The ratio $\frac{CO_2 \text{ dissolved}}{CO_2 \text{ combined}}$ calculated for different periods in the experiment would in each case indicate an increased C_H of the blood. It is of interest, however, to note there was slight fluctuation in this factor at different periods. The fact that it was higher in the first instance than in any other would suggest that increased lung ventilation was counteracting the acidotic process due to anæsthesia.

As stated above this method is not beyond criticism, but the further application of it may furnish useful information.

SUMMARY.

(1) The rate of elimination of water, acid, and basic phosphates and ammonia by the kidney in periods before, during and after anæsthesia has been determined in a number of cases.

(2) The acidity of the urine, as estimated by this method, is not materially altered during surgical anæsthesia.

(3) A decrease in the C_H of the blood during surgical anæsthesia is not indicated.

(4) The acidity of the urine may be decreased during anæsthesia by the induction of hyperpnœa.

It is suggested that during surgical anæsthesia two processes, which are mutually antagonistic, are active, one the "acidotic process" of Haggard and Henderson due directly to disturbance in metabolism as a result of anæsthesia, the other the over-ventilation process.

Whether the C_H of the blood falls, rises or remains unchanged during surgical anæsthesia would therefore depend upon the relative intensities of these two processes.

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THE INFLUENCE OF THE REACTION OF MEDIA AND OF THE PRESENCE OF BUFFER SALTS ON THE METABOLISM OF BACTERIA.

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THE physical chemical ideas of neutrality, and the character of so-called buffer salts have been associated for some considerable time with physiological problems involving the chemistry of respiration and its connection with the behaviour of blood. It is only a fairly recent development that the bacteriological conceptions of alkalinity and acidity have been diverted from results obtained by titration with an indicator to a so-called "neutral point," to those where the neutral point has been that given by the hydrogen electrode or by indicators, the changing-point of whose range is definitely known.

The influence of buffer substances on growth has also been imperfectly understood. The content of these substances varies enormously in different media used by the bacteriologist, and may range from a practically negligible quantity in the synthetic media used by Galimard, Winogradsky and others to strong solutions of peptones or meat media, where a very considerable amount of acid may be produced in the medium by the metabolism of bacteria without appreciably changing the true reaction.

It will not be necessary to go into the theoretical considerations involved in the use of buffer salts, for these have been fully reviewed in previous work by Henderson, whose paper with Webster, published some twelve years ago, is one of the first real contributions concerning the influence of the reaction on growth.

More recently Clark and his co-workers, Cole and Onslow, Wyeth and Svanberg, have furnished us with important data concerning the influence of the reaction of the medium on the growth of bacteria, so that bacteriologists are now commencing to replace the older notions of alkalinity with those which have a physico-chemical basis.

The present set of experiments was undertaken with a practical object which was of considerable urgency during the war.

In the preparation of toxins for immunisation, considerable difficulty was experienced in obtaining a product of high titre. The batches of toxins varied in toxicity and were stable for a comparatively short time, and then only in the cold. Certain information derived from other members of the Anaërobe Committee of the Medical Research Council indicated that the initial reaction of the medium played an important part in the production of a potent toxin. It seemed also that the "fragility" of the toxin on keeping might be connected with the final reaction of the medium, and that the qualities of added buffer salts might be profitably employed to protect the medium from a too acid final reaction.

It is also possible that in the preparation of bacterial emulsions for vaccines the reaction of the medium might have an influence on the immunising quality of the bacteria themselves. This phase of vaccine production has not, so far as I am aware, been subjected to critical investigation.

As Harris has shown, a marked constancy in metabolism can be obtained when an organism is allowed to ferment in a medium of constant composition. It was therefore determined to compare the effect of bacteria fermenting in the same medium with and without a buffer salt. An opportunity was taken of comparing the action of various buffer salts on bacterial metabolism. We know from the work of Sörensen, to whom we owe the real initial impetus to this type of investigation, that the range in which the buffering by salts takes place is an individual quality, so that it would be obviously useless to employ a salt whose buffer range was on the acid side of any supposed critical point in fragility.

On the other hand, it might be useful to start a fermentation at a point considerably more alkaline than the buffer range, and to allow the fermentation to come to the range in which the buffer acted and be kept there for as long a time as possible.

Certain difficulties are encountered in doing this. Some bacteria will not tolerate more than a certain concentration of salts, such as sodium phosphate, and if the amount present be increased above an amount which seems to be pretty narrowly defined, growth is totally inhibited. In any attempt to prevent the reaction of a medium becoming too acid, one is therefore confronted, on the one hand, by the toxicity of the buffer salt, and on the other by the fact that, with a low concentration of these salts, the medium is rendered rapidly acid through the products of metabolism.

EXPERIMENTAL DATA.

As a standard medium the "tryptic broth" digest of Cole and Onslow was employed in all these experiments. This medium has a very constant amino-acid and ammonia content, and for that reason can be safely employed as a substrate.

Only one sugar, glucose, was used. The experiments ought to be repeated

with other sugars, because one cannot safely undertake to apply the results obtained with a mono-saccharide to fermentations in which a di-saccharide is the carbohydrate used.

As buffer salts, three were employed. Of these, sodium phosphate, used in the form of the partially dehydrated di-hydrate, occupied the middle place, buffering strongly between P_H 6 and P_H 7·5. On the acid side of this potassium phthalate was used. This absorbs hydrogen-ions with the production of a flat curve between P_H 6 and P_H 4·5. Sodium pyrophosphate was used in the alkaline region of P_H 8. This salt, which, so far as I am aware, has not hitherto been used for this purpose, deserves some attention from bacteriologists who desire a buffered medium with an initial reaction of about P_H 8.

This salt has a formula of $Na_4P_2O_7$, and may change by the action of acids to either the orthophosphate or to the half saturated acid sodium pyrophosphate $Na_2H_2P_2O_7$. It is probably in the change from the tetra-sodium salt to the di-sodium di-hydrogen salt that the buffer action of the compound takes place.

The technique used was essentially that described by Harris in his paper on the comparison of the metabolism of *B. sporogenes* and the "Reading" bacillus.

Two hundred c.c. of medium were employed from which adequate quantities of gas could be secured for analysis. The fixed acids were determined by extracting 100 c.c. of the medium in a continuous ether extraction apparatus of my own design, but which is in many respects similar to the independently constructed apparatus of Koolmann. The extractions were usually carried out for about eighteen hours. After extraction, the ether was evaporated off, the residue taken up in about 500 c.c. of water, and boiled over a free flame until reduced in bulk to about 50 or 75 c.c. In this way practically all the volatile acids are driven off. There may be a slight loss of fixed acids (Harden, Wolf), but this was neglected. The acid solution is treated with an excess of standard alkali and titrated back with acid, using phenolphthalein as an indicator. The process for the determination of volatile acids has been previously described.

PRELIMINARY EXPERIMENT.

The experiments described were not done in the order in which they are presented. The first organism used was *Vibrion septique*. As the practical urgency of the problem was lessened some time after the commencement of the experiments *B. coli* was selected, as owing to the work of Harden, Theobald Smith, Keyes and Gillespie and Clark, much more is generally known about the fermentative characteristics of this bacillus. The experiments with *B. coli* are therefore presented first, and those with *B. sporogenes*, *Vibrion septique* and *B. welchii* follow.

As, however, the first experiments were of an urgent nature, and the results were suggestive, one of these is given in detail.* It affords some information regarding time relationships not found in later experiments.

The idea was that part of the falling off in toxicity of the toxin of *Vibrion septique* was due to the increasing acidity of the medium as the result of the metabolism of the organism. In order to prevent this in a more effective way than by adding calcium carbonate, a certain quantity of the di-sodium hydrogen phosphate was added to the medium. This buffers strongly in the region of P_H 7·2. The salt used was the di-hydrate. The amount of phosphate added is not without consequence. When 5 per cent. of the salt is added to a peptone bouillon containing 0·2 per cent. of glucose and the medium is inoculated with *V. septique*, fermentation is considerably delayed. The resulting toxin is less potent than when smaller quantities of the buffer are used. Metabolism does take place, for considerable quantities of acids are formed. The reaction of the medium changes only slightly. Experiments showed that *V. septique*, when grown in peptone bouillon to which 0·2 per cent. glucose had been added, produces volatile acids equivalent to about 20 c.c. of a decinormal acid solution per 100 c.c. of medium. If 20 c.c. of decinormal hydrochloric acid be added to peptone bouillon containing 2 per cent. of the di-hydrated di-sodium phosphate, the reaction changes from P_H 7·1 to P_H 6·91. Hence this concentration of the salt should be ample to buffer the medium. That this is the case the following experiment will show :

A stock medium was prepared, consisting of—

Casein digest (tryptic broth)	333 c.c.
Double strength Lemco extract	270 „
Sodium chloride 0·5 per cent.	297 „

This medium was adjusted to a reaction of P_H 7·3. Fifteen hundred c.c. of this medium were placed in Winchester quart bottles and arranged for fermentation purposes as described by Wolf and Harris.

To one of the bottles 30 grm. of sodium phosphate were added ; the other was left without the buffer salt. After sterilisation a quantity of sterile glucose solution was added, sufficient to make the contents of each flask equivalent to 0·2 per cent. of the sugar.

For inoculation the " Stokes " strain of *V. septique* was used, 10 c.c. from a cooked meat culture being used as the inoculum in each case. The organism had been prepared by animal passage through a guinea-pig, the muscle being planted in peptone bouillon, and a single plating made from the culture. A single colony was picked off and planted into cooked meat, which culture was used directly for inoculating the experimental culture. At intervals readings of the gas production were made, samples taken for analysis, and experiments performed to determine the toxicity.

* In the earlier experiments I was assisted by Mr. I. E. J. Harris, B.A., B.Sc., to whom it is a great pleasure to acknowledge my thanks.

An examination of the table reveals that while the production of gas is much less in the phosphated medium, the amounts of ammonia and volatile acids in the two fermentations are practically the same. The constancy of the reaction of the buffered medium is continued longer in the buffered than in the unbuffered medium. The curve of ascent of acidity is less steep.

The most notable difference is in the potency of the two toxins so produced. Samples were taken at 24 hours, 48 hours and 96 hours. These were centrifuged at high speed, and the supernatant liquid injected into the marginal ear veins of rabbits.

The results may be tabulated as follows:

Plain Medium.

Hours.	Dose.	Weight (lbs.).	Death.	Time.
24	0·5	2·25	+	18 hours
48	0·5	2·25	+	1 hour
72	0·25	?	0	—

Phosphated Medium.

Hours.	Dose.	Weight (lbs.).	Death.	Time.
24	0·5	2·25	+	18 hours
48	(*a*) 0·5	2·25	+	6 mins.
	(*b*) 0·5	6	+	6 ,,
72	(*a*) 0·25	3·5	+	2 ,,
	(*b*) 0·125	4·5	—	—
96	(*a*) 0·25	4	+	12 ,,
	(*b*) 0·125	4·25	—	—
240	0·25	5	+	10 ,,
528	0·5	6	—	—

One c.c. of a 5 per cent. phosphate bouillon solution when injected into rabbits caused no inconvenience.

It will thus be seen, from the above experiments, that it was possible to retain the toxicity of a peptone meat-extract toxin of *V. septique* at 37° C. for ten days with a high potency till the end of this period. Miss Robertson has been able to prepare a highly toxic product by inoculating plain broth or glucose broth medium which has been adjusted to a reaction of P_H 7·8–8 with bits of infected liver of guinea-pigs dead of *V. septique* infection. It is possible that this method allows the formation of toxin, and enough time to elapse before the medium becomes sufficiently acid to permit a toxin destruction.

The analytical results shown in the following table are instructive. They show that metabolism has proceeded in the buffered and unbuffered media very much to the same extent, except in two characteristics. One of these is the gas formation; the other is the true reaction.

In the case of the "plain" medium there was an abundant evolution of

gas, amounting to 448 c.c. in 24 hours. Less than one quarter of this volume of gas was evolved by the phosphated medium. In the same length of time the "plain" medium had reached its highest acidity, while the phosphate culture had only increased from $P_H = 7{\cdot}3$ to $P_H = 6{\cdot}84$. It will, however, be noticed that the total amount of gas from the phosphate medium when the experiment was continued for 144 hours was about four-tenths of that yielded by the unbuffered medium. The final value at the end of 96 hours was only $P_H = 6{\cdot}68$. It is worthy of especial note in this connection that the volatile acid production in the two media was identical.

No. of hours.	Gas: c.c. per litre.	Total NH_2N: mgrm. per 100 c.c.	NH_3N formed: mgrm. per 100 c.c.	Total NH_3N: mgrm. per 100 c.c.	NH_3N formed: mgrm. per 100 c.c.	Reaction P_H.	Total volatile acids: c.c. N/10 sol. per 100 c.c.	Volatile acids formed: c.c. N/10 sol. per 100 c.c.
			0·2 *per cent. Glucose-peptone-bouillon.*					
0	0	94	0	24	0	6·9	6	0
24	448	95	1	29	5	5·45	21	15
48	533	—	—	34	10	5·76	21	15
72	574	—	—	34	10	5·82	21·6	15·6
96	595	—	—	36	12	5·82	23·6	17·6
144	604	—	—	36	12	5·87	—	—
240	654	—	—	37	13	5·92	27	21
			0·2 *per cent. Glucose-peptone-bouillon plus* 2 *per cent.* Na_2HPO_4.					
0	0	89	0	22	0	7·3	6	0
24	106	93	4	28	6	6·84	19	13
48	174	—	—	32	10	6·77	22	16
72	193	—	—	34	12	6·75	22	16
96	203	—	—	34	12	6·68	22	16
144	211	—	—	—	—	—	—	—
240	—	—	—	36	14	6·67	31·6	25·6
528	—	—	—	—	—	6·8	—	—

The main points which will be noticed in the effect of phosphates on the general character of the metabolism of *V. septique* are the following: There is at the end of 144 hours nearly three times as much gas formed by the non-buffered medium as in that to which phosphate has been added. The features of the nitrogen metabolism, as represented by the ammonia production, are substantially the same in the two media, and the amount of volatile acids is alike. In the reaction, however, there is a marked difference.

While in the unbuffered medium at the end of 24 hours one has reached the highest acidity represented by $P_H = 5{\cdot}45$, in the buffered medium the reaction has become more acid only by a change represented by a rise of

hydrogen-ion concentration from $P_H = 7{\cdot}3$, the initial reaction, to $P_H = 6{\cdot}84$. Allowing the bacteria to ferment for a further 216 hours produced a subsequent rise to only P_H 6·67. Doubling the time caused what seems to have been a slight reversion, the hydrogen-ion concentration falling to P_H 6·8 at the end of 528 hours. The main features, therefore, of this experiment are that gas production is depressed by the addition of phosphates, while ammonia formation and volatile acid formation are not greatly influenced. The addition of the buffers has a decided influence on the final reaction of the medium, preventing the increase in acidity of the final product.

Reilly, Hichinbottom, Henley and Thaysen describe the influence of calcium carbonate on the production of acetone from maize mash. Here the attempt to keep the medium in the neighbourhood of neutrality is accompanied by the almost complete disappearance of *n*-butyl alcohol and acetone from the final product, while the formation of acetic and butyric acids was greatly increased.

Obviously the process in which phosphates maintain the medium in the neighbourhood of the neutral point is quite a different one from that observed by Reilly and his co-workers when using calcium carbonate.

EXPERIMENTS WITH *B. COLI*.

The organism used in these experiments was the H strain employed by Wyeth in this laboratory in his study of the effects of acids on the growth of this organism.

A preliminary test was made to ascertain the amount of phosphate which would be necessary to inhibit fermentation in plain peptone, and in a peptone medium to which glucose to a concentration of 2 per cent. had been added. The highest concentration of Na_2HPO_4 expressed as the anhydrous salt was 3 per cent.

The results were as follows:

Per cent. Na_2HPO_4.	Plain.		Glucose.	
	Gas in 6 hrs.	P_H 23 hrs.	Gas in 6 hours.	P_H 23 hours.
3	?	7·7	+	6.5
2	+	7·5	++	6·1
1·5	++	7·5	++	5·6
1	++	7·3	++	5·2
0·5	++	7·2	+++	5·2

Gas was formed in 3 per cent. phosphate in plain peptone in 23 hours, but there was a distinct slowing of the gas evolution, and it was decided on the strength of this experiment not to exceed a concentration of sodium phosphate of 2 per cent.

The series of experiments performed with *B. coli* were as follows:

(1) The effect of sodium phosphate on media containing varying concentrations of glucose.

(2) Following these were experiments in which a salt, sodium pyrophosphate, buffering in an alkaline region was used.

(3) Experiments with a salt, acid potassium phthlate, buffering in an acid region.

The experiments will be discussed together, and the individual points may be seen by a reference to the tables printed at the end of the paper.

With *B. coli* the experiments include media containing 0·5 per cent. glucose (Experiments B and F), and media containing 1 per cent. glucose (Experiments D and H). The concentration of sodium phosphate was in each case 2 per cent. reckoned as $Na_2HPO_4 . 2H_20$. As controls, similar media containing the same concentration of carbohydrate, but without the buffer salt, were fermented at the same time and under the same conditions of inoculum and temperature. These controls are fermentations A, C, E and G. A and C, and E and G are the same media, as will be seen from the analytical results.

As the glucose was added after sterilisation and the phosphate had but little effect on the composition of the nitrogen constituents of the media, the nitrogen partition of the buffered medium may be taken as that of the unbuffered.

Sodium Phosphate Experiments.

An examination of the gas evolution induced by *B. coli* reveals a similar situation to that seen with *V. septique.*

In Experiments A and B, in which a concentration of 0·5 per cent. glucose was used, the addition of phosphate retarded gas formation ; at the fifth hour only 6 c.c. had been evolved in the buffered medium, while 91 c.c. were obtained from the plain solution. At the tenth hour 197 c.c. and 81 c.c. were obtained respectively. The final readings, taken about the twenty-ninth hour, showed a difference between 245 c.c. and 178 c.c.

This difference was more pronounced when the concentration of glucose was increased to 1 per cent. Very little more gas was produced from the phosphated medium than when 0·5 per cent. of sugar was employed, while in the unbuffered solution the effect of increasing the amount of sugar was plainly seen in increased gas production. This difference continued up to the eleventh hour. At this point the phosphate medium commenced to increase its gas production, and at the twenty-sixth hour the two media had produced gas within 13 c.c. of one another.

In Experiments E and F, where fermentation was continued for 48 hours with a 0·5 per cent. solution of glucose, one finds that the increasing time allows the phosphated solution to produce nearly the same amount of gas as the medium without the salt.

The results of Experiments G and H are interesting and instructive. Here 1 per cent. glucose was employed and the fermentation was continued for 36 hours. Up to the sixth hour the production of gas in the phosphate medium had lagged behind. From that time on it gathered speed. At 24 hours the plain medium had produced its maximum amount of gas,

284 c.c., while at that time 375 c.c. were produced in the phosphated medium. This amount was further increased to 387 c.c. at the thirty-sixth hour.

It seems that the phosphates mainly influence gas production in its time relation. A very well-marked lag is effected. In two media of similar composition, while the earlier quantities of gas are smaller when phosphate is employed, the total quantity may be considerably larger if the fermentation is prolonged. The reason for this is that fermentation is inhibited early in the plain medium by the production of acid and gas production ceases.

In the phosphated solution protection is afforded from increasing hydrogen-ion concentration, and consequently gas production continues for a longer time and in increasing quantity.

The effect of phosphates on the composition of the evolved gases in this series of experiments is quite uniform. In all cases relatively more hydrogen is evolved when phosphates are present in the medium. In connection with this, as one might expect, there is an increased production of both volatile and fixed acids in the phosphate medium, except in one instance (Experiments E and F), where the fixed acid production is lower in the phosphated medium. There was insufficient material to repeat these analyses.

It seems clear, however, that phosphates influence the carbohydrate metabolism of *B. coli* in increasing gas and acid production, and to a certain extent in modifying the qualitative character of the reaction. An examination of the volatile acid products, as was done in the case of *B. welchii* by Wolf and Telfer, would throw some light on this matter.

With regard to the effect of phosphates on the nitrogen metabolism, although one has noted a very obvious difference in the toxicity of buffered and non-buffered *V. septique* cultures, one does not find any indication in the coarse processes as studied from ammonia and amino-acid determinations. It would seem that rather more ammonia is produced, but the differences in the amino-acid analyses are such that it would be difficult to draw any plausible conclusions.

The effect of adding phosphates on the final reaction of the medium is shown in experiments C and D. Where the final reaction in a 1 per cent. glucose peptone medium rose to P_H 4·68 in 26 hours, the reaction of the the medium to which phosphate had been added rose only to P_H 6·3, although nearly twice as much volatile acids had been formed from the glucose.

The Influence of Sodium Pyrophosphate on the Metabolism of B. coli.

As has been mentioned, Miss Robertson and others have found increased potency of the toxins prepared when the starting-point of the medium was more alkaline than P_H 8. The usual mixtures used in preparing buffer solutions in this region are those containing borates, which are unsuitable for introducing into media. It seems that sodium pyrophosphate might be suitable for this purpose. Unfortunately no titration curves have been made

with this salt, but a 2 per cent. concentration of this salt will produce a reaction of $P_H + 8$. Preliminary experiments showed that it was not toxic in concentrations of this amount. As we already had sufficient data regarding the unbuffered media, two experiments were performed in which the concentrations of sugar were 1 per cent. and 2 per cent. respectively.

The composition of the gas varies considerably between the two experiments, the higher concentration of carbon dioxide occurring with the higher concentration of glucose.

The final reactions of the two experiments show that 2 per cent. of sodium pyrophosphate is insufficient to buffer the amount of volatile and fixed acids produced in a 2 per cent. glucose medium. The amount of volatile and especially of fixed acids is very much greater than was observed in any of the other experiments. In the 2 per cent. glucose fermentation, the hydrogen-ion concentration had risen to P_H 4·80, which is in the neighbourhood of the acid inhibiting point. Although volatile acids corresponding to 40·2 c.c. of N/10 alkali, and fixed acids corresponding to 45·6 c.c. of N/10 alkali were produced in the 1 per cent. glucose solution, the hydrogen-ion concentration had only risen to P_H 6·08 from P_H 8·24. It would appear, therefore, that sodium pyrophosphate may serve a very useful purpose where a buffer salt is required for bacteriological media when a low hydrogen-ion concentration is desired.

The Effect of Potassium Phthalate on the Metabolism of B. coli.

The "reaction resultants" of *B. coli* in media containing phthalates have been studied in detail in my laboratory by Wyeth, and this salt had previously been used by Clark in his study of *B. coli*. No work has been done on the metabolism of organisms grown in a medium containing the salt. It was realised at the outset that its buffer qualities were confined to a rather high hydrogen-ion concentration, and that the drop from an initial hydrogen-ion concentration of $P_H \pm 7·5$ would be sudden, as no buffering takes place at this point. The fermentation was continued for 47 hours, and the concentrations of glucose were the same as in the preceding experiments, with which it can usefully be compared.

The gas production falls somewhat behind that of the pyrophosphate fermentations, and the relative concentration of carbon dioxide is between the two figures obtained there.

The volatile acid production corresponds with what was found in using phosphates as a buffer, but appears to be slightly lower than what is obtained with sodium pyrophosphate. Owing to the presence of phthalic acid the fixed acid production could not be ascertained.

There is nothing noteworthy in the nitrogen metabolism. The figures for ammonia production and amino-acid consumption are well within the limits found in the preceding experiments.

EXPERIMENTS WITH *B. WELCHII.*

The experiments performed with *B. welchii* are in many respects similar to those done with *B. coli.* It will not be necessary to discuss them in detail, but only the principal points will be noted, especially in so far as they show a difference from the facultative anaërobe. Any special details may be obtained from the tables themselves.

Fermentations were carried out with media containing 2 per cent. of sodium phosphate with 0·5 per cent. glucose (Experiments S and T) and with 1 per cent. glucose (Experiments Q and R). The main point in the experiments with *B. welchii* is the effect of phosphates on gas production. While these salts delayed the evolution of gas by *B. coli*, it was found that when the experiment was continued long enough, nearly the same amount of gas was produced from the phosphated medium. This is not the case with *B. welchii.* Not only is the gas production delayed, but the amount is never so large. In the Experiments Q and R for example, 564 c.c. of gas were evolved from the plain medium in 9 hours, while only 14 c.c. of gas were evolved from the same volume of phosphated medium. In Experiments S and T the plain medium had produced 293 c.c. of gas in 52 hours, while from the phosphated medium only 7 c.c. were obtained. After a fermentation lasting 219 hours 167 c.c. of gas were evolved, while the amount from the plain medium was 314 c.c.

The phosphate had a distinct effect on the composition of the evolved gas, the carbon dioxide content of which rose to 66·2 per cent. It is, however, difficult to see any evidences of a difference in the direction of the nature of the reaction being shifted from an examination of the volatile or fixed acid fermentation. Both these products are formed to the same extent in the plain medium as in the phosphated.

The nitrogen metabolism does not show any changes which will account for the differences in the gas production. In other words, carbohydrate and nitrogen metabolism, in so far as the analytical results go, do not disclose the reason for the very profound difference in gas formation due to the presence of phosphates. A more complete examination of the products of fermentation is required.

EXPERIMENTS WITH *VIBRION SEPTIQUE.*

The experiments recorded here may be discussed shortly, as the results obtained are in practically every respect similar to those obtained in the larger fermentations described at the commencement of the paper. They enlarged these results in so far as beef extract was excluded from the medium, and the concentration of glucose was varied from 0·5 per cent. to 1 per cent.

The most marked feature of these experiments is the suppression of the gas production when the experiment is not too long continued. Thus in Experiments AE and AF, when the fermentation lasted 156 hours, only 7 c.c. of gas were detected in a 0·5 per cent. glucose peptone medium, while the

control had reached its maximum in 77 hours with 235 c.c. An even more striking example is furnished by Experiments AG and AH, where 1 per cent. glucose was used. With an evolution of 432 c.c. of gas by the control fermentation in 156 hours, 3 c.c. were evolved by the phosphate medium—an amount which is within the limits of experimental error.

A survey of the analytical results would make it appear that little metabolism was taking place, and this is more especially the case in volatile acid formation. Except in experiments AB, and especially experiment V, there is no evidence of volatile acid formation. (In V there has been gas formed to the extent of 41 c.c.) But in all cases metabolism has taken place as judged by the fixed acids produced. That viable organisms were present in every case was ascertained by sub-inoculations.

It is a matter of practical interest that no potent toxin was ever obtained in any of these experiments. Whether the use of meat extract is absolutely necessary for this purpose must be the subject of future investigation.

EXPERIMENTS WITH *B. SPOROGENES*.

As all the foregoing experiments were carried out with essentially saccharolytic organisms, it was necessary to complete the series by an examination of the effect of buffer salts on two proteolytic organisms. For this purpose *B. sporogenes* (Metchnikoff) and *B. histolyticus* were chosen. These had been the subject of previous study by Harris and myself. The concentrations of glucose employed varied from 0·5 per cent. to 2 per cent.

The effect of phosphates on fermentation by *B. sporogenes* are shown in the lag in gas production in the shorter experiments (AN and AR). In one experiment, AP, where the concentration of glucose was 2 per cent., this lag was not seen, and the amount of gas produced by the phosphated medium was always in excess of that formed by the "plain."

The nitrogen metabolism as gauged by the ammonia and amino-acid production is not altered to any remarkable extent by the introduction of buffer salts into the medium. On the whole neither the amino-acid production nor the ammonia production are quite as high as in the plain medium, but the differences are so small that it is not possible with the number of experiments performed to make any definite statement.

The volatile acid production seems to be slightly depressed, while the fixed acid is increased in quantity by the introduction of phosphates. No decisive change takes place in the composition of the evolved gases.

It will be noted that, as in the previous experiments, the addition of phosphates has influenced the final reaction of the media, the phosphate fermentation showing a lower hydrogen-ion concentration. As the growth of *sporogenes* does not tend to a very acid end-point, the differences between the two are not great.

EXPERIMENTS WITH *B. HISTOLYTICUS*.

The series of experiments concludes with fermentations of *B. histolyticus*—an organism which is essentially proteolytic. The chemical activities were studied by Wolf and Harris (1918), but no fermentations in glucose-peptone were carried out. It only forms very large quantities of gas when fermented in cooked meat, and the composition of the gas is distinguished by being almost completely carbon dioxide. Three experiments were performed with this organism, the concentrations of glucose varying between 0·5 and 2 per cent. It is noticeable that, although the amount of gas evolved is very small, phosphates act in the same direction as with saccharolytic types of organisms. The amount in a phosphate medium is always below that of the plain.

The effect on nitrogen metabolism is distinct. The ammonia formation is high in both media, but the production in the presence of phosphates is always less. In two of the experiments there was a consumption of the amino-acid portion of the medium. In the third (AW and AX) the amount of amino-acids had increased as the result of fermentation.

The acid production was not influenced to any great extent by the presence of a buffer salt; the fixed acids seem to be slightly increased in consequence.

Owing to the amount of ammonia produced in fermentation the final hydrogen-ion concentration is low, but in all cases increase in acidity was inhibited by the presence of phosphates.

SUMMARY.

The effect of moderate concentrations of di-sodium hydrogen phosphate (2 per cent.) on the growth of *B. coli*, *Vibrion septique*, *B. welchii*, *B. sporogenes* and *B. histolyticus* in glucose-peptone has been investigated. The effect of acid potassium phthalate and sodium pyrophosphate on the growth of *B. coli* was also ascertained.

In all cases the buffer salt either partially inhibits gas production or produces a lag. If the fermentation be very prolonged, the volume of gas may finally approach that obtained from a non-buffered medium. The effect of phosphates on gas production is most pronounced in the case of actively saccharolytic organisms, but is also definite with proteolytic bacteria such as *B. sporogenes* and *B. histolyticus*.

The effect of phosphates on the nitrogen metabolism is not pronounced. On the whole the formation of amino-acids and ammonia from a medium containing peptones, polypeptides and amino-acids is not increased.

In the case of the saccharolytic organisms the acid production appears to be increased when buffer salts are added to the medium. This bears out some of the results which have been obtained with similar organisms when fermentation takes place in the presence of calcium carbonate.

The final reaction of the fermentations is affected by the presence of buffer salts, in the sense that in no case did the medium become so acid in the presence of buffer salts as it did when these salts were absent.

The toxin formation in *V. septique* cultures is influenced by the presence of a buffer salt—sodium phosphate. The toxin had a higher potency than the control made without the salt. It is shown that toxins may be kept potent for long periods of time in the presence of sodium phosphate under conditions which would quickly destroy the toxicity of a product made in an unbuffered medium.

TABLES.

TABLE I.—*Experiments A and B.* B. coli *in* 0·5 *per cent. Glucose-peptone with and without* 2 *per cent. Sodium Phosphate.*

	A. Plain.		B. Phosphate.	
	Initial.	Final.	Initial.	Final.
Total nitrogen per cent.	0·412	0·385	0·440	0·407
Ammonia nitrogen: mgrm. per 100 c.c.	22·4	33·3	22·4	39·5
Amino-acid nitrogen: mgrm. per 100 c.c.	138	141	137	118
Volatile acids: c.c. N/10 acid per 100 c.c.	3·8	23·6	5·05	34·6
Fixed acids: c.c. N/10 acid per 100 c.c.	—	36·2	—	37·4
Carbon dioxide per cent.	—	51·3	—	37·4
Hydrogen per cent.	—	48·7	—	62·6
Total gas: c.c. in 28·6 hours	—	245	—	178
P_H 20° C.	7·03	5·36	7·42	6·91

*Gas Production.**

Hours.	c.c. A.	c.c. B.
3·4	22	5
5·4	102	10
12	213	100
23·25	237	163
28·4	245	178

TABLE II.—*Experiments C and D.* B. coli *in* 1 *per cent. Glucose-peptone with and without* 2 *per cent. Sodium Phosphate.*

	C. Plain.		D. Phosphate.	
	Initial.	Final.	Initial.	Final.
Total nitrogen per cent.	0·412	0·398	0·440	0·406
Ammonia nitrogen: mgrm. per 100 c.c.	22·4	33·3	22·4	36·2
Amino-acid nitrogen: mgrm. per 100 c.c.	138	112	137	112·5
Volatile acids: c.c. N/10 acid per 100 c.c.	3·8	25·7	5·05	46·3
Fixed acids: c.c. N/10 acid per 100 c.c.	—	29·7	—	?
Carbon dioxide per cent.	—	52·1	—	41·6
Hydrogen per cent.	—	47·9	—	58·4
Total gas: c.c. in 26 hours	—	30·2	—	28·9
P_H 20° C.	7·03	4·68	7·42	6·30

* In order to save space only a few points are given out of a large number of observations.

Gas Production.

Hours.	c.c. C.	D.
3·4	35	5
5·4	130	16
12	256	270
23·25	297	283
28·4	302	289

* TABLE V.—*Experiments I and J.* B. coli *in* 1 *per cent. and* 2 *per cent. Glucose-peptone with* 2 *per cent. Sodium Pyrophosphate.*

	I. 1 per cent. glucose.		J. 2 per cent. glucose.	
	Initial.	Final.	Initial.	Final.
Total nitrogen per cent.	0·357	0·332	0·357	0·317
Ammonia nitrogen: mgrm. per 100 c.c.	15	28·1	15	23·4
Amino-acid nitrogen: mgrm. per 100 c.c.	118	98·5	118	89·2
Volatile acids: c.c. N/10 acid per 100 c.c.	4·7	40·2	4·7	32·6
Fixed acids: c.c. N/10 acids per 100 c.c.	—	45·6	—	82
Carbon dioxide per cent.	—	45·9	—	53·2
Hydrogen per cent.	—	54·1	—	46·8
Total gas: c.c. in 49 hours	—	405	—	445
P_H 20° C.	8·24	6·08	8·24	4·80

Gas Production.

Hours.	c.c. 1 per cent. I.	2 per cent. J.
3	2	1
24	86	422
28·3	244	438
31·15	301	441
49	405	445

TABLE VI.—*Experiments O and P.* B. coli *in* 1 *per cent. and* 2 *per cent. Glucose-peptone with* 2 *per cent. Acid Potassium Phthalate.*

	O. 1 per cent. glucose.		P. 2 per cent. glucose.	
	Initial.	Final.	Initial.	Final.
Total nitrogen per cent.	0·381	0·380	0·381	0·36
Ammonia nitrogen: mgrm. per 100 c.c.	17·8	29·5	17·8	27·2
Amino-acid nitrogen: mgrm. per 100 c.c.	130	110	130	103
Volatile acids: c.c. N/10 acid per 100 c.c.	7·1	27·8	7·1	30·2
Carbon dioxide per cent.	—	51·3	—	51·3
Hydrogen per cent.	—	48·7	—	48·7
Total gas: c.c. in 47 hours	—	406	—	402
P_H 20° C.	+8	5·48	+8	5·25

* At the request of the Editors, the author has allowed Tables III, IV, XI, XIV and XVII to be omitted.

Gas Production.

Hours.	1 per cent. O. (c.c.)	2 per cent. P. (c.c.)
7·4	1	91
8·2	2·6	134
8·7	21	166
9·2	39	186
11·2	113	242
23	292	402

TABLE VII.—*Experiments Q and R.* B. welchii *in* 1 *per cent. Glucose-peptone with and without* 2 *per cent. Sodium Phosphate.*

	Q. Plain. Initial.	Q. Plain. Final.	R. Phosphate. Initial.	R. Phosphate. Final.
Total nitrogen per cent.	0·345	—	0·333	0·308
Ammonia nitrogen: mgrm. per 100 c.c.	19·6	36·4	19·8	34·5
Amino-acid nitrogen: mgrm. per 100 c.c.	114	108	112	111
Volatile acids: c.c. N/10 acid per 100 c.c.	4·2	40·3	6·4	46·7
Fixed acids: c.c. N/10 acid per 100 c.c.	—	14	—	11
Total gas: c.c. in 9 hours	—	564	—	14
P_H 20° C.	7·1	>4·4	7·4	5·9

Gas Production.

Hours.	Q. (c.c.)	R. (c.c.)
3	55·8	3
5	168	4
7	255	7
9	297	14

TABLE VIII.—*Experiments S and T.* B. welchii *in* 0·5 *per cent. Glucose peptone with and without* 2 *per cent. Sodium Phosphate.*

	S. Plain. Initial.	S. Plain. Final.	T. Phosphate. Initial.	T. Phosphate. Final.
Total nitrogen per cent.	0·356	0·398	0·334	0·354
Ammonia nitrogen: mgrm. per 100 c.c.	20·9=	55·4	22·1	41·2
Amino-acid nitrogen: mgrm. per 100 c.c.	128	174·4	116	148
Volatile-acids: c.c. N/10 acids per 100 c.c.	7·7	36·9	16·2	34·3
Fixed acids: c.c. N/10 acids per 100 c.c.	—	31·7	—	28·9
Carbon dioxide per cent.	—	49·7	—	33·8
Hydrogen per cent.	—	50·3	—	66·2
Total gas: c.c. in 219 hours	—	314	—	167
P_H 20° C.	7·01	6·10	7·32	6·8

Gas Production.

Hours.	S. (c.c.)	T. (c.c.)
1·25	5	5
8	6	5
22·5	263	6
52	293	7
219	314	167

TABLE IX.—*Experiments U and V.* V. septique *in* 1 *per cent. Glucose-peptone with and without* 2 *per cent. Sodium Phosphate.*

	U. Plain.		V. Phosphate.	
	Initial.	Final.	Initial.	Final.
Total nitrogen per cent.	0·345	0·315	0·333	0·282
Ammonia nitrogen: mgrm. per 100 c.c.	19·6	22·3	19·8	21·9
Amino-acid nitrogen: mgrm. per 100 c.c.	114	107	112	110
Volatile acids: c.c. N/10 acid per 100 c.c.	4·25	25·9	6·4	33·9
Fixed acids: c.c. N/10 acid per 100 c.c.	—	4·8 ?	—	17·6
Total gas: c.c. in 30 hours	—	138	—	41
P_H 20° C.	7·1	4·8 ?	7·4	6·3

Gas Production.

Hours.	U. (c.c.)	V. (c.c.)
3	9	10
5	9	11
8	21	5 ?
24	124	32
30	138	41

TABLE X.—*Experiments Y and Z.* V. septique *in* 0·5 *per cent. Glucose-peptone with and without* 2 *per cent. Sodium Phosphate.*

	Y. Plain.		Z. Phosphate.	
	Initial.	Final.	Initial.	Final
Total nitrogen per cent.	0·356	0·393	0·334	0·355
Ammonia nitrogen: mgrm. per 100 c.c.	20·9	26·9	22·1	26·9
Amino-acid nitrogen: mgrm. per 100 c.c.	128	137·6	116	134·4
Volatile acids: c.c. N/10 acids per 100 c.c.	7·7	23·9	16·2	30·7
Fixed acids: c.c. N/10 acid per 100 c.c.	—	28·5	—	27·9

	Y. Plain.		Z. Phosphate.	
	Initial.	Final.	Initial.	Final
Carbon dioxide per cent.	—	42·6	—	40·5
Hydrogen per cent.	—	57·4	—	59·5
Total gas: c.c. in 219 hours	—	121	—	105
P_H 20° C.	7·01	5·10	7·32	6·23

Gas Production.

Hours.	c.c. Y.	Z.
1·25	6	8
8	6	9
22·5	23	26
52	50	6?
73	120	55
219	121	105

TABLE XII.—*Experiments AE and AF.* V. septique *in* 0·5 *per cent. Glucose-peptone with and without* 2 *per cent. Sodium Phosphate.*

	AE. Plain.		AF. Phosphate.	
	Initial.	Final.	Initial.	Final.
Total nitrogen per cent.	0·413	0·402	0·442	0·418
Ammonia nitrogen: mgrm. per 100 c.c.	22·5	28·3	4	22·4
Amino-acid nitrogen: mgrm. per 100 c.c.	139·1	143·3	127	141
Volatile acids: c.c. N/10 acid per 100 c.c.	3·8	25·6	5	4·2
Fixed acids: c.c. N/10 acid per 100 c.c.	—	25·1	—	10·4
Carbon dioxide per cent.	—	41·2	—	—
Hydrogen per cent.	—	58·8	—	—
Total gas: c.c. in 156 hours	—	234	—	7
P_H 20° C.	7·01	5·05	7·32	7·32

Gas Production.

Hours.	c.c. AE.	AF.
23	119	4
33·7	160	4
49	205	3
77	235	9
135	234	7
156	234	7

TABLE XIII.—*Experiments AG and AH.* V. septique *in* 1 *per cent. Glucose-peptone with and without* 2 *per cent. Sodium Phosphate.*

	AG. Plain.		AH. Phosphate.	
	Initial.	Final.	Initial.	Final.
Total nitrogen per cent.	0·413	0·401	0·442	0·432
Ammonia nitrogen: mgrm. per 100 c.c.	22·5	28·6	22·4	23·4
Amino-acid nitrogen: mgmr. per 100 c.c.	139·1	141·8	127	132·6
Volatile acids: c.c. N/10 acid per 100 c.c.	3·8	34·1	5	4·2
Fixed acids: c.c. N/10 acid per 100 c.c.	—	26·6	—	9·5
Carbon dioxide per cent.	—	41·6	—	—
Hydrogen per cent.	—	58·4	—	—
Total gas: c.c. in 156 hours	—	432	—	—
P_H 20° C.	7·01	5·17	7·32	7·40

Gas Production.

Hours.	Gas c.c. AG.	AH.
23	233	3
27	253	3
49	370	3
70	415	3
113	431	3
135	430	1
156	432	3

TABLE XV.—*Experiments AO and AP.* B. sporogenes *in* 2 *per cent. Glucose-peptone with and without* 2 *per cent. Sodium Phosphate.*

	AO. Plain.		AP. Phosphate.	
	Initial.	Final.	Initial.	Final.
Total nitrogen per cent.	0·336	0·344	0·368	0·347
Ammonia nitrogen: mgrm. per 100 c.c.	11·2	50	18·4	43·9
Amino-acid nitrogen: mgrm. per 100 c.c.	142	148	131	149
Volatile acids: c.c. N/10 acid per 100 c.c.	4·8	40·2	5·9	48·9
Fixed acids: c.c. N/10 acid per 100 c.c.	—	11·5	—	13·6
Carbon dioxide per cent.	—	64·2	—	60·9
Hydrogen per cent.	—	35·8	—	39·1
Total gas: c.c. in 94 hours	—	415	—	440
P_H 20° C.	7	5·6	7·3	5·8

Gas Production.

Hours.	AO. (c.c.)	AP. (c.c.)
1·5	2·1	4·2
23	153	197
28·5	201	250
47	312	400
71	400	420
94	415	440

TABLE XVI.—*Experiments AQ and AR.* B. sporogenes *in* 1 *per cent. Glucose-peptone with and without* 2 *per cent. Sodium Phosphate.*

	AQ. Plain. Initial.	AQ. Plain. Final.	AR. Phosphate. Initial.	AR. Phosphate. Final.
Total nitrogen per cent.	0·356	0·383	0·334	0·366
Ammonia nitrogen: mgrm. per 100 c.c.	20·9	68·7	22·1	65·8
Amino-acid nitrogen: mgrm. per 100 c.c.	128	154	116	138
Volatile acids: c.c. N/10 acid per 100 c.c.	7·7	44·4	16·2	63·3
Carbon dioxide per cent.	—	64·4	—	65
Hydrogen per cent.	—	35·6	—	35
Total gas: c.c. in 78 hours	—	262	—	166
P_H 20°C.	7·01	6·17	7·32	6·44

Gas Production.

Hours.	AQ. (c.c.)	AR. (c.c.)
5	6	8
7	6	8
22	132	19·4
28	179	48·9
47	228	129
78	262	166

TABLE XVIII.—*Experiments AU and AV.* B. histolyticus *in* 2 *per cent. Glucose-peptone with and without* 2 *per cent. Sodium Phosphate.*

	AU. Plain. Initial.	AU. Plain. Final.	AV. Phosphate. Initial.	AV. Phosphate. Final.
Total nitrogen per cent.	0·368	0·363	0·336	0·367
Ammonia nitrogen: mgrm. per 100 c.c.	18·4	75·2	11·2	70·2
Amino-acid nitrogen: mgrm. per 100 c.c.	131	119	142	130
Volatile acids: c.c. N/10 acid per 100 c.c.	5·9	41·8	4·8	50
Fixed acids: c.c. N/10 acid per 100 c.c.	—	3·3	—	9·9
Total gas: c.c. in 94 hours	—	49·5	—	32·6
P_H 20° C.	7·01	6·7	7·32	7

Gas Production.

Hours.	c.c. AU.	AV.
1·5	2·1	1
23	13·7	14·7
28·5	18·9	20
47	24·7	26·8
71	27·4	25·2
94	49·5	32·6

TABLE XIX.—*Experiments AW and AX.* B. histolyticus *in* 1 *per cent. Glucose-peptone with and without* 2 *per cent. Sodium Phosphate.*

	AW. Plain.		AX. Phosphate.	
	Initial.	Final.	Initial.	Final.
Total nitrogen per cent.	0·356	0·394	0·334	0·35
Ammonia nitrogen: mgrm. per 100 c.c.	20·9	84·5	22·1	51
Amino-acid: nitrogen mgrm. per 100 c.c.	128	138	116	126
Volatile acids: c.c. N/10 acid per 100 c.c.	7·7	59·1	16·2	26·6
Total gas: c.c. in 78 hours	—	35·7	—	14·2
P_H 20° C.	7·01	6·38	7·32	7·2

Gas Production.

Hours.	c.c. AW.	AX.
5	1	3·1
7	3·1	4·2
22	10	5·8
28	14·2	5·8
47	25·2	11·5
78	35·7	14·2

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GLYCOGENIC INFILTRATION OF THE LIVER OF LABORATORY RABBITS.

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In March, 1919, I was asked by the Salvarsan Committee of the Medical Research Council to report upon the histological changes in material obtained from rabbits which had been subjected to experiment.

Two groups of experiments, A and B, had been made. In each of these groups one series of rabbits had been inoculated with 0·85 per cent. saline made with contaminated water, whilst another parallel series had been inoculated with salvarsan dissolved in a 0·85 per cent. saline made with the same contaminated water. For convenience I shall employ "W." (water) to represent contaminated saline alone, and "S." to represent salvarsan in contaminated saline. The two types of experiment in each series of the groups A and B can thus be represented by "W. A," "S. A," and "W. B," "S. B," whilst each pair of experiments can be distinguished by the addition of a numeral, *e.g.* "W. A II," "S. A II," "W. B IV," "S. B IV."

The material consisted of microscopic sections of the liver from nineteen experiments, of the kidney from twenty, of the myocardium from seventeen and of the spleen from fifteen. All the tissues enumerated had been embedded in paraffin and had been stained with hæmatoxylin and eosin; there were also a few sections of livers treated with osmic acid, and one frozen section of a liver stained with Sudan III and hæmatoxylin. I was informed that the tissues had been fixed in formaldehyde solution.

HISTOLOGICAL CHANGES.

A comparison of the sections from different cases showed that the most striking differences were exhibited by the parenchyma of the *liver*.

In certain livers there was a very conspicuous vesiculation of the cytoplasm of the hepatic cells, associated with changes in, and absence of, nuclei. I arranged the livers, therefore, on two occasions at a week's interval in series according to the estimated degrees of cytoplasmic vesiculation. On each

occasion six groups were separated. The two estimations, however, showed some differences in the order of the individual livers in each group. The subdivision of the groups was, therefore, abandoned.

Group 1.—In W. A IV and S. B III the parenchyma showed no greater vesiculation than is seen in all paraffin sections of the liver. A few nuclei were stained abnormally faintly.

Group 2.—In W. A VI, S. A VII, W. B III, S. B VI and S. B VII the cytoplasm was interrupted by small, clear clefts and spaces. These were frequently perinuclear. In W. A VI and S. A VII the cells in the periphery of the lobules were affected more than those in the centre. In W. B III, S. B VI and S. B VII the central cells were somewhat more affected than the peripheral. Faint staining of nuclear chromatin and absence of nuclei were seen in all; these nuclear changes varied in amount from "slight" to "considerable."

Group 3.—In W. A III, S. A III, and W. B II there was slightly greater vesiculation, and the dissociated cytoplasm was decidedly granular. Faint staining of nuclei was considerable.

Group 4.—In W. A II and S. A II vesiculation was considerably greater, and the cytoplasm was conspicuously granular. In both cases more cells in the centres of the lobules were affected than in the periphery. In both cases faint staining of nuclei was considerable, and was found especially in central cells.

Group 5.—In W. A I vesiculation was much greater, and, in the centres of the lobules, caused a somewhat different appearance. The cells in the central third or more of the lobules were greatly swollen and either round or cubical. These cells were conspicuously vesicular, part of the cytoplasm forming a distinct border, or membrane, to the cell-body, whilst the remainder was scattered in the form of granules, generally united to form a net, throughout a colourless, clear ground. Differences in nuclear structure, and apparent nuclear degeneration indicated by extreme pallor of nuclei and absence of nuclei, were greater than in the previous sections.

Group 6.—In W. B I, W. B IV, W. B VI, S. B I, S. B II and S. B IV the vesiculation was of the type described in the previous paragraph, but was considerably greater. The cells were greatly swollen, and the cytoplasm was almost entirely reduced to a border round the clear cell. The hepatic columns frequently resembled sections of hollow spheres, or hollow cubes, piled in rows, some showing a nucleus, placed centrally, but many failing to do so. Variations in structure, size and in the depth of coloration of the nuclei, and absence of nuclei were greater than in the other livers. The extreme pallor of many nuclei was a conspicuous feature. In W. B IV the maximum degree of vesiculation seldom affected more than the central one-third or two-thirds of the lobules. In W. B I, W. B VI and S. B IV the central two-thirds were chiefly affected. In S. B I the cells in the extreme periphery of the lobules

were sometimes free. In S. B II great vesiculation was present throughout the lobules, but was greater in the intermediate and peripheral zones.

The following table gives the estimated degrees of vesiculation of the hepatic cells, expressed by numbers of + signs, in each series of the two groups of experiments A and B.

TABLE I.

W. A I + + + + +	S. A I (no section).
W. A II + + +	S. A II + + +.
W. A III + +	S. A III + +.
W. A IV −	S. A IV (no section).
W. A VI +	S. A VI (no section).
W. A VII (no section)	S. A VII +.
W. B I + + + + + + +	S. B I + + + + + + +.
W. B II + +	S. B II + + + + + +.
W. B III +	S. B III −.
W. B IV + + + + + + +	S. B IV + + + + + +.
W. B VI + + + + + + +	S. B VI +.
W. B VII (no section)	S. B VII +.

The livers also showed various degrees of portal infiltration and fibrosis. These changes were associated with cholangitis. They were exactly similar to the inflammatory changes which accompany coccidiosis. In S. A III and S. A VII there were granulomatous nodules such as are seen in coccidiosis, and in S. A II and S. B VII a few oöcyst capsules of *Eimeria stiedæ*, surrounded by giant-cells, were present within bile-ducts. A table comparing the degree of parenchymatous vesiculation with the degree of portal infiltration and fibrosis showed that there was no trace of correlation between the two conditions. There can be no doubt that the portal infiltration and fibrosis were due to coccidiosis, an infection from which, in my experience, very few laboratory rabbits have been free.

In the *kidneys* there was evidence of parenchymatous degeneration. I made a comparative estimate of the degree of degeneration, employing nuclear degeneration as the criterion for differentiation. I found this to be a matter of great difficulty, there being no such conspicuous differences as were present in the livers. A table comparing the estimated degrees of parenchymatous vesiculation in the livers with the degrees of parenchymatous degeneration in the kidneys showed that there was no correlation between the two conditions.

In six kidneys (S. A III, S. A IV, W. B I, W. B VI, S. B III and S. B VI) there were small focal areas of infiltration. These lay either in the cortex and upper medulla, or in the cortex and intermediate zone, or in the intermediate zone and medulla, or in the intermediate zone alone or in the medulla alone.

Within these areas round and small polygonal, mononuclear cells, occasionally accompanied by spindle-cells, lay in the interstitial tissue, and there was a catarrhal desquamation of the epithelium in the included tubules. The presence of this infiltration bore no relation to the estimated amount of vesiculation of the hepatic parenchyma. On the other hand, these focal inflammatory areas occurred, with one exception, within kidneys in which the degree of parenchymatous degeneration had been estimated to be greatest.

The *myocardium and spleen* did not show any changes of significance.

THE NATURE OF THE CHANGES IN THE HEPATIC PARENCHYMA.

In all the sections of the liver stained with hæmatoxylin and eosin there was a vesiculation of the cytoplasm of the hepatic cells. The livers have been grouped above (p. 311) according to the degrees of this vesiculation.

In Group I the vesiculation was very slight, and, indeed, no greater than is found in all sections of liver embedded in paraffin. In the other groups the vesiculation, judged by morphological characters, which alone can be the test in sections stained with hæmotoxylin and eosin, was similar in kind but showed great variations in degree. The vesiculation was associated with nuclear changes, the estimated frequency and degree of the alterations in the nuclei corresponding closely to the degree of vesiculation of the cells.

Morphologically the vesiculation differed in certain details from that which is seen in paraffin sections when the cytoplasm is affected by fatty infiltration or degeneration. Further, in the few preparations specially stained for the demonstration of fat no fat was shown. Again, I had previously seen a vesiculation, morphologically similar to that in Group 6, in the centres of the hepatic lobules of three rabbits. Two of these rabbits had been given trinitrotoluene. The third was sent to me as a normal control: it was not in reality normal, because there were in the kidneys numerous hyaline and hæmorrhagic casts, and calcification of many cells lining convoluted tubules. In the livers of these three rabbits only very occasional small, fatty granules were demonstrated by Sudan III. It was therefore possible to exclude fatty change as the cause of the vesiculation.

The vesiculation was not that of "hyaline droplet degeneration," because the granules floating in the vacuolar cell-bodies had neither the size, definite spherical shape nor deep eosinophilia of "hyaline droplets."

The lesser grades of the vesiculation and granulation (in Groups 2, 3 and 4) resembled closely in morphology those which express "cloudy swelling" or "albuminous degeneration" in paraffin sections. I considered, therefore, that the great vacuolation seen in Groups 5 and 6 might represent a severe degree of "dropsical degeneration," a condition which I believe to differ from "albuminous degeneration" only in a greater absorption of water by the cytoplasm. The extreme pallor of many of the nuclei and the absence of other nuclei

appeared to demonstrate karyolysis, and thus supported the view that the vesiculation was an expression of degeneration.

It appeared strange, however, that such extreme degrees of dropsical degeneration should be present in the liver, and should bear no relation to the degree of parenchymatous degeneration in the corresponding kidneys. Moreover, the changes in the cells in the most severely affected livers reminded me of those seen in cells which I had proved to be loaded with glycogen, for instance in cells lining tubules of the kidney in cases of diabetes and in cells of Grawitz's carcinoma of the kidney. I considered it advisable, therefore, to ascertain the effect of glycogenic infiltration on the liver of the rabbit.

Prof. James McIntosh, at that time working in the department of Prof. William Bulloch, kindly put an apparently normal rabbit at my disposal and assisted me in an experiment. A large bunch of carrots was placed in the rabbit's hutch at ten in the morning. Four hours later the animal was killed instantly by a single blow on the head, and within half a minute of death small portions of the liver had been excised and dropped into absolute alcohol; the alcohol was immediately heated until it boiled. Other portions of the liver were then excised from the margins of the previous excisions, and were placed into 4 per cent. saline formaldehyde solution.

A necropsy on the rabbit revealed to the naked eye no abnormalities except a few necrosed, coccidial areas in the liver and one softened mesenteric gland. Portions of the lungs, kidneys, myocardium, thyroid gland, pancreas, spleen, suprarenal bodies and ovary were fixed in 4 per cent. saline formaldehyde and were embedded in paraffin. Sections were stained with Ehrlich's hæmatoxylin and eosin, Weigert's iron-hæmatoxylin and van Gieson's mixture, and Weigert's fuchselin and neutral red. No abnormalities were detected on microscopic examination.

The portions of liver fixed in boiling alcohol were embedded in celloidin, and sections were stained by Best's carmine method for the demonstration of glycogen. One of the portions fixed in formaldehyde was cut upon the freezing microtome, and sections were stained in Sudan III and hæmatoxylin. The remaining portions were embedded in paraffin, and sections were stained in hæmatoxylin and eosin, etc.

In the sections stained with Sudan III no fat was demonstrated, except in the endothelial cells of Kupffer, in one or two hepatic cells, in the form of a few small granules, and in a few cells of a portal infiltration, which was connected with the coccidiosis.

In the sections stained by Best's method the hepatic cells were loaded with glycogen (Plate-fig. 2).

The appearance of the cells in the material fixed in formaldehyde, embedded in paraffin and stained with hæmatoxylin and eosin is represented in Plate-fig. 1. These sections resembled very closely those of Group 6 above. The majority of the cells most severely affected by the vesiculation lay in

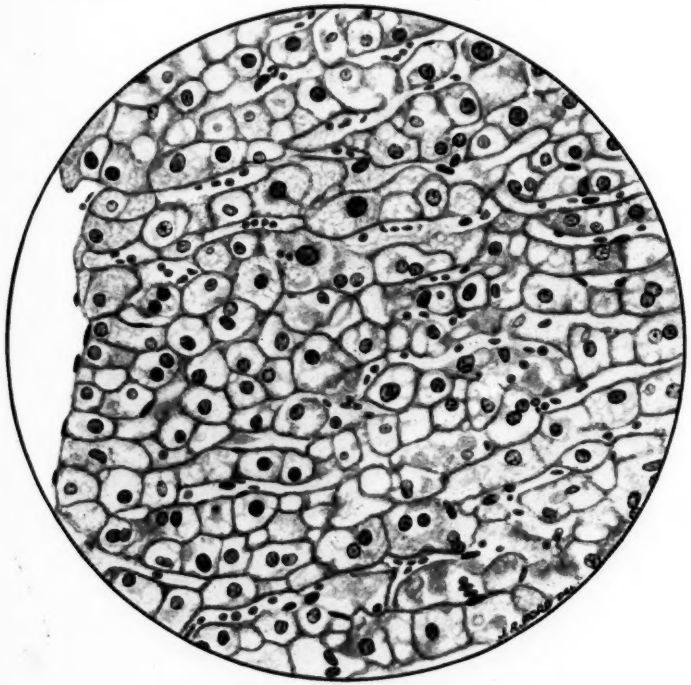

FIG. 1.—Liver of rabbit. Formaldehyde; paraffin; hæmatoxylin and eosin. × 335.

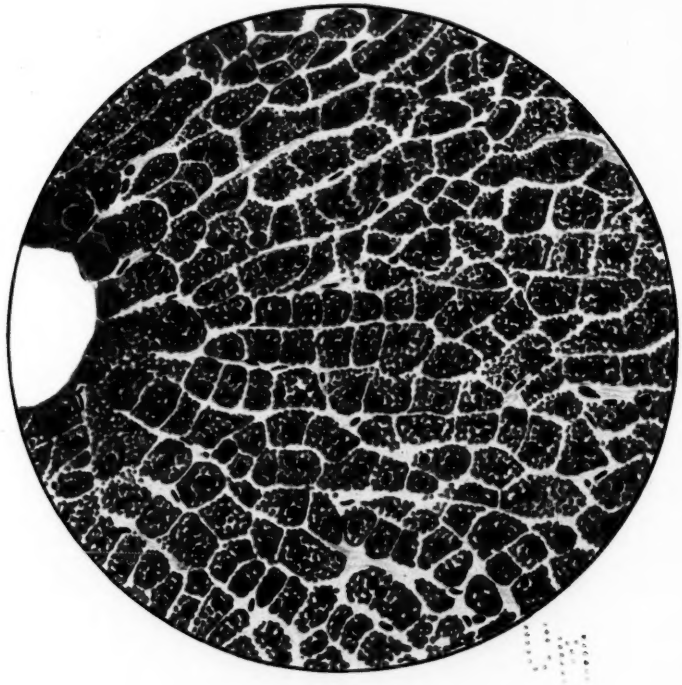

FIG. 2.—Same liver. Alcohol; celloidin; Best's glycogen stain. × 335.

the central zones of the lobules. The nuclei showed great variation in size, in structure and in affinity for hæmatoxylin. In many cells no nucleus was present. Sir Frederick Andrewes, Prof. Bulloch, and my colleagues, Dr. G. B. Bartlett and Dr. W. W. Woods, kindly examined these sections and the sections of Groups 5 and 6. They all expressed the opinion that the changes were identical in nature.

It was impossible to doubt, therefore, that in the livers of the rabbits which had been inoculated with contaminated saline or with contaminated saline and salvarsan, the vesiculation and nuclear changes were expressions of a glycogenic infiltration such as can occur in a healthy rabbit after a suitable meal. It was impossible to regard these changes as evidence of experimentally induced degeneration.

The variations in the structure and staining affinities of the nuclei of the cells in which glycogenic infiltration was proved to be present were evidently expressions of physiological nuclear activity. Only a few types of these variations are included in the two fields selected for illustration. Some of the variations in the size of the nuclei and the complete absence of nuclei could be ascribed to thin sections of greatly swollen cells; in a thin section a swollen cell might be cut external to its nucleus or through the periphery of its nucleus. The fact that absence of nuclei appeared to be more frequent in the thin paraffin sections than in the relatively thick frozen and celloidin sections confirms this. There were, however, cells without nuclei in thick sections. Further, in all sections there were small nuclei, frequently of irregular outline, so faintly stained as to be just recognisable. The appearances suggested, therefore, that karyolysis and complete loss of nuclei must be included among the changes which may occur during physiological activity of hepatic cells.

The single feeding experiment, described above, indicates a simple method of determining the phases through which the nucleus of the hepatic cell passes during glycogenic metabolism. To point out this line of research is not, however, the primary object of this paper. My object is to warn those who experiment upon animals against mistaking this physiological process for a pathological degeneration. Table I shows that in four out of seven experiments, in each of which two rabbits were employed, the estimated degree of vesiculation corresponded exactly in each pair of animals; that is to say, in four out of seven pairs of animals the liver was in the same stage of physiological activity. In experiments such as these, in which one animal is employed as a control to the other, the stage of hepatic metabolism might well have corresponded in every pair of experiments; the greater the care taken in selecting pairs of animals of like age and nutrition, and in ensuring that both animals were kept under exactly similar conditions, the more likely would this have been to occur. Had this occurred in the experiments under discussion, and had the vesiculation and nuclear changes been accepted as a pathological phenomenon, then the conclusion would have been inevitable that the con-

taminated water had caused degeneration of the liver, and that the addition of salvarsan to the water had not affected the result.

In conclusion, I thank the Salvarsan Committee for permitting me to publish this paper, and the Medical Research Council for defraying the expense of the reproduction of the beautifully executed paintings by Mr. J. R. Ford.

SUMMARY.

The histological changes seen in ordinary microscopic preparations of the rabbit's liver when infiltrated with glycogen after a suitable meal resemble closely those of a severe parenchymatous degeneration. The danger of mistaking in animal experiment these physiological changes for manifestations of degeneration is illustrated by an account of the findings in a series of experiments in which pairs of rabbits were inoculated respectively with contaminated water and with salvarsan dissolved in contaminated water. Incidentally attention is drawn to the promising field offered by glycogenic infiltration of the liver of the rabbit for the experimental study of changes in the nucleus during physiological activity of the hepatic cell.

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